1988

Application of supported enzymes in clinical chemistry.

Erica. Sullivan
University of Windsor

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, tests publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

APPLICATIONS OF SUPPORTED ENZYMES IN CLINICAL CHEMISTRY

by

ERICA (FORSYTHE) SULLIVAN

A Major Clinical Chemistry Critique
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements
for the Degree of Master of Science at the
University of Windsor

Windsor, Ontario
1988

ISBN 0-315-43773-1

ABSTRACT

The basic principles of enzyme immobilization, methods of immobilization and effects of this technology on the enzyme are discussed. The applications and advances of this technology in the clinical chemistry laboratory, particularly over the past ten years, for the analytes glucose and urea is reviewed.

Experimental data for the development of an analytical method to assay for functional group density on a nylon support matrix used in enzyme immobilization are reported. Near quantitative recoveries, from the hydrolysis of the imidate salt of nylon formed from alkylation with triethyloxonium tetrafluoroborate, could be achieved in 0.1 M glycine buffer, pH 9.0. This study shows that using alcohol oxidase and peroxidase and the chromogen system of 4-aminoantipyrene and sodium 2-hydroxy-3,5-dichlorobenezenesulfonate, an assay could be developed for measuring ethanol liberated from the intentional hydrolysis of the imidate salt of the nylon.

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. K.E. Taylor, for his patient direction and encouragement during the course of this work.

I would also like to thank Dr. R.J. Thibert for his guidance and helpful suggestions.

My appreciation also extends to the other members of my committee: Drs. T. Hyde and D.A. Cotter for their reading of this critique.

I would also like to thank my friends, especially J.G. and E.P. in the Department of Chemistry and Biochemistry for their moral support and companionship.

Finally, I would like to thank my husband Rob for the many ways in which he has helped throughout this study.

DEDICATION

TO MY HUSBAND

ROB

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

4-AAP	4-Aminoantipyrene
ADH	Alcohol dehydrogenase
ALT	Alanine aminotransferase
AO	Alcohol oxidase
BSA	Bovine serum albumin
GO	Glucose Oxidase
HDCBS	Sodium 2-hydroxy-3,5-dichlorobenzenesulfonate
Km	Michaelis constant
LDH	Lactate dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
POD	Horseradish peroxidase
TEOTFB	Triethyloxonium tetrafluoroborate
Tris	Tris (hydroxymethyl)aminomethane
Vmax	Maximum reaction velocity

CHAPTER I

INTRODUCTION

A. SUPPORTED ENZYMES

The use of enzymes in analytical chemistry has made great advances over the past several years. However, the relatively high cost and limited stability of enzymes has slowed efforts to fully exploit these biocatalysts. The development of techniques which allowed enzymes to be supported on, or immobilized to a polymeric matrix, such that they could be utilized repeatedly without loss of catalytic activity, has circumvented many of these problems.

1. Definition of Immobilization

A "supported" or "immobilized" enzyme has been defined by Trevan (1) as an enzyme molecule imprisoned in a distinct phase that allows exchange with, but is separated from the bulk phase in which substrate, effector or inhibitor molecules are dispersed and monitored. According to Klibanov (2), immobilization is the conversion of enzymes from a water soluble, mobile state to a water insoluble immobile state. Goldstein (3) has defined immobilized enzymes as those physically confined or localized in a certain defined region or space with retention of their catalytic activities, which can be used repeatedly and continuously.

Since a supported or immobilized enzyme is one whose

mobility is restricted within a limited region of space, the term covers any enzyme that is constrained through micro-encapsulation, gel entrapment, adsorption and covalent binding as well as any combination and modification of these.

2. Merits and Drawbacks of the Technique

The immobilization of an enzyme results in several advantages. As alluded to earlier, the immobilized enzyme offers the analytical laboratory the advantages of reusability and stability. Once immobilized, an enzyme is often stable for weeks or months (4), thereby enabling use of some enzymes that in soluble form were considered too unstable for practical use. In addition, by immobilizing, spontaneous association between proteins which can result in autolysis and aggregation can usually be prevented.

Since an immobilized enzyme may be separated from the solution phase, the final product may be free of enzyme. The enzyme can then be collected after catalysis is completed and reused to achieve many more analyses than could be performed with the same amount of enzyme in solution. This technology can give rise to analytical procedures that are much simpler and more reliable than classical methods and readily lend themselves to use in automated analysis (5). The immobilized enzyme can be incorporated into a cylindrical flow-through device, to be used in a continuous enzyme

reactor (IMER), in which a continuous flow of substrate enters at one end and a continuous flow of products emerges at the other end (6).

Immobilization, like any method that may affect protein structure, is not free of certain drawbacks. As with any chemical modification, immobilization may change the conformation of an enzyme compared to that in solution. Since the enzyme when immobilized, is very often bound chemically to a support, the enzyme derivative is in fact, a different chemical species from the native protein and may have quite different catalytic properties. There may be a number of changes in pH optimum, temperature of heat denaturation and substrate specificity (7). In addition, non-specific interactions, namely, hydrophobic, electrostatic, and hydrogen-bonding, between the enzyme and support may occur. The support can also present itself as a barrier to free diffusion of molecules to and from the enzyme, generating concentration gradients of substrate and product that do not exist in a soluble homogeneous solution.

3. Effects of Immobilization on the Enzyme

The fundamental characteristics of an enzyme catalyzed reaction are usually changed in some manner by immobilization. The nature of the change is dependent on the inherent properties of the enzyme, and additional characteristics imposed by the support material on the enzyme, substrate or

product, as well as the method of immobilization. Most investigations have focused on the evaluation of the kinetic parameters, particularly Km, the effect on pH activity profiles and on the stability of bound enzymes.

(a) Stability

Several types of stability (i.e., ability to resist alteration), can be considered: resistance to inactivation by heat, disruption by chemicals, digestion by proteases, inactivation by change in pH, loss of activity during storage or loss of activity due to process operations.

Generally, it is found that immobilization (particularly covalent immobilization), improves an enzyme's resistance to heat, chemical disruption and pH changes (8). This is likely achieved by providing extra rigidity to the folded protein chain and therefore greater resistance to unfolding.

In the analytical sense, storage and operational stability is of foremost interest. The operational stability which is a function of the enzyme, the carrier durability, pH and inhibitor concentrations in the reagent stream and the analyte solutions, is often increased. In the majority of cases, storage stability which will provide the investigator some idea of the shelf-life of the reagent, is also expected to increase.

The stability of a preparation can also be affected by the electrostatic charges on the support itself. This may

result in partitioning of substrates, products or inhibitors towards or away from the matrix surface, thus concentrating or depleting these species in the immediate vicinity of the enzyme (9).

(b) Activity

Immobilization almost invariably changes the catalytic activity of an enzyme. This effect has been investigated mainly by comparing the activity of bound enzyme with that of native enzyme in solution. In view of the wide variety of enzymes and procedures employed, generalizations are difficult. According to most studies, the maximum reaction velocity (Vmax) obtained with a supported enzyme is usually lower than that obtained with the corresponding soluble enzyme (6). The Michaelis constant (Km), which reflects the affinity that the enzyme has for its substrate, is usually changed, indicating that the active site of the enzyme may be altered, thereby affecting binding of substrate. The constants obtained, therefore, are apparent values.

Immobilized enzymes generally show lower specific activity than the native enzyme (10). The decrease in activity is often attributed to conformational changes in the enzyme structure or to steric hindrances in the immediate vicinity. Covalent immobilization is most likely to alter the protein conformation.

The reaction rate is also reduced by diffusional

limitations; as the substrate is consumed, more substrate must diffuse into the enzyme phase from the bulk solution. This is normally a problem for all forms of immobilized enzymes, but especially for encapsulated enzymes (8). This effect can be minimized by using low enzyme concentrations, high flow rates, and, when applicable, small diameter tubing.

B. SCOPE OF THE STUDY

This critique is divided into five chapters. CHAPTER I provides an introduction to the subject of enzyme immobilization, while CHAPTER II will discuss the major types of immobilization methods, particularly those involving covalent modification. CHAPTER III will review the applications of supported enzyme technology in Clinical Chemistry. The literature particularly over the past 10 to 15 years will be examined. Emphasis will be placed on the immobilization methods used for the determination of the analytes glucose and urea and a summary will be provided for analytes.

Although it may be perceived that techniques such as enzyme-linked immunosorbent assay (ELISA), which involves the enzyme's immobilization during the course of analysis may be a candidate for this study, techniques involving antibodies are beyond the scope of this discussion. Instead, the critique will begin with a discussion of immobilized enzyme electrodes, and continue with the applications of

immobilized enzymes in reactor form.

The penultimate chapter will serve to further characterize covalent methods of immobilization. This chapter, will deal with some experiments designed to determine the functional group density on nylon tubing intended for enzyme coupling, after activation with the alkylating agent triethyloxonium tetrafluoroborate (TEOTFB). Experimental details will be given and the chapter will close with results of the investigation and a discussion of the significance of these results. Lastly, CHAPTER V, will summarize and provide conclusions of the results of the experimental section, as well as consider their relevance to the principles discussed in the body of the critique.

CHAPTER II

METHODS OF IMMOBILIZATION

This chapter will provide a synopsis of the chemical aspects of enzyme immobilization, as well as describe the methods most commonly used for the fixation of enzymes onto solid supports.

In contrast to the isolation and purification of an enzyme, for which often just one optimal method applies, the same cannot be said for the immobilization technique and the support chosen for a particular enzyme. This is because the usefulness of an immobilized enzyme preparation will depend on its particular area of application, whether in an industrial process, in medicine, as a biological model system or as an analytical reagent.

The majority of the methods available for the immobilization of enzymes can be grouped into 4 main classes:

A. Adsorption on inert supports or ion-exchange resins.

B. Entrapment, by occlusion within cross-linked gels or by encapsulation within a microcapsule.

C. Cross-linking by bi- or multi-functional reagents, often followed by adsorption or entrapment.

D. Covalent binding to polymeric supports, preferably via functional groups nonessential for the biological activity of the protein.

The strategy employed when immobilizing an enzyme, involves not only the means of immobilization but also a choice of an appropriate support. Additional functional groups may have to be introduced to achieve the desired physicochemical properties on the polymer and the carrier must not only be stable, but should also possess a suitable geometrical shape and appropriate dimensions (11).

A. ADSORPTION

Adsorption of an enzyme onto a support material is the simplest and most economical method for immobilizing an enzyme on a support. Basically, it is the adhesion of an enzyme to a support material that has not been specifically functionalized for covalent attachment (12). Adsorption of an enzyme can be achieved by simply mixing an aqueous solution of enzyme with the support material for a period of time, after which the excess enzyme is washed away from the immobilized enzyme.

Depending on the nature of the surface, the binding forces between the enzyme and the support may be the result of ionic interactions, physical adsorption, hydrophobic interaction or hydrogen bonding (1). The characteristics of these forces are such that changes in the pH, temperature, ionic strength, concentration of enzyme and adsorbent and the presence of an organic solvent may have a significant effect on the efficiency of the immobilization.

Numerous surface-active materials have been used in the preparation of enzyme-adsorption complexes. Some of the most popular being ion-exchange resins, activated charcoal, silica gel, clays, alumina, and controlled-porosity glasses and ceramics in a variety of physical shapes, i.e., sheets, fibers, beads, etc. A suitable adsorbent should have high affinity and capacity for the enzyme and should not adsorb the reaction product or enzyme inhibitors.

A major drawback of this technique when based mainly on electrostatic attraction to charged supports, lies in the tendency of such conjugates to dissociate upon increasing the ionic strength or varying the pH or the temperature of the medium. Some of these disadvantages can be overcome by increasing the charge on the protein by chemical modification (13). Moreover, because the binding is non-covalent, a certain amount of enzyme leakage can occur from the immobilized enzyme preparation. In addition, nonspecific adsorption has been shown in a number of cases to give rise to partial or total inactivation (6). Adsorption techniques are thus of limited reliability when irreversible immobilization of an enzyme is desired.

B. ENTRAPMENT AND ENCAPSULATION

Immobilization by entrapment is based on the occlusion of enzyme within a constraining structure tight enough to prevent protein from diffusing into the surrounding medium,

while still allowing penetration of the substrate(s) and departure of the product(s) (10).

Entrapment of an enzyme may be achieved by mixing an enzyme with a polymer material and then cross-linking the polymer to form a lattice structure that traps the enzyme. Alternatively, the enzyme can be mixed with chemical monomers that are then polymerized to form a cross-linked polymeric network, trapping the enzyme in the interstitial spaces of the lattice (1). The latter method is more widely used. The porosity of the gel lattice or semi-permeable membrane is controlled to ensure that the structure is tight enough to prevent leakage of enzyme and at the same time allow free movement of substrate and product. The obvious advantage is their generality, since the enzyme molecule itself does not participate directly in the formation of the water-insoluble constraining structure. This method is limited by the fact that it is suitable mainly for enzymes that utilize substrates of molecular weights low enough to pass through the matrix. In addition, diffusional resistances to the penetration of substrate usually lead to perturbed kinetics manifested as low specific activities for the immobilized enzyme (14). Furthermore, the amount of enzyme that can be entrapped is limited by the solubility of the enzyme in the entrapping reaction. The most popular matrices for gel entrapment include polyacrylamide, silicone rubber, polyvinyl alcohol, starch and silica gel (9).

A method of entrapment by a bead-polymerization procedure similar to the preparation of cross-linked polyacrylamide has been described (15). In this procedure, an aqueous solution containing enzyme and acrylic monomers is dispersed in a hydrophobic phase and polymerized, resulting in well defined spherical beads. The beads contain entrapped active enzyme and show good mechanical stability and high flow rates in column processes.

Guilbault and Das (16) have prepared immobilized cholinesterase and urease on silicone rubber but have reported that the relatively low yield of active enzyme was obtained due to the rigorous polymerization conditions. In addition, the hydrophobic nature of the polymer may likely restrict the permeability of the water-soluble substrate.

The advantages of gel entrapment include the experimental simplicity, and the variety of forms in which the gels can be used. The disadvantages include the control of numerous experimental factors, the possible inactivation of the enzyme by the radicals necessary for polymer formation, and the restriction of the size of the substrate and products to relatively small species.

A related technique, i.e., encapsulation, can be achieved by enveloping enzyme molecules within various forms of semi-permeable membranes. Large proteins or enzymes cannot pass out of or into the capsule, but small substrates and products can pass freely across the semi-permeable

membrane. Materials such as nylon, cellulose nitrate and biological cells have been used to construct micro-capsules varying from 10 μm to 1000 μm in diameter (8).

C. CROSS-LINKING

Supported enzyme derivatives have been prepared by cross-linking by methods using only the enzyme or those which use the enzyme and a carrier protein such as albumin (1). The common cross-linking reagents are shown in Figure 1. Immobilization of enzymes, solely by cross-linking into large aggregates, has found limited application because of the difficulties encountered in controlling the intramolecular cross-linking while obtaining a high degree of intermolecular cross-linking (9). In addition, the bifunctional reagent may preferentially attack the active site of the enzyme, thus rendering it inactive. The single advantage of the method is that a single reagent can be used to prepare numerous enzyme derivatives. Of the many cross-linking reagents that have been used only glutaraldehyde has found extensive use (1). Glutaraldehyde, is a bifunctional aldehyde used to react with polymers containing primary amino groups, resulting in the formation of an aldehyde function for protein binding.

Another approach has been in the preparation of "hybrid" immobilized enzyme systems, where coupling occurs after adsorption onto a water insoluble matrix or as a

FIGURE 1

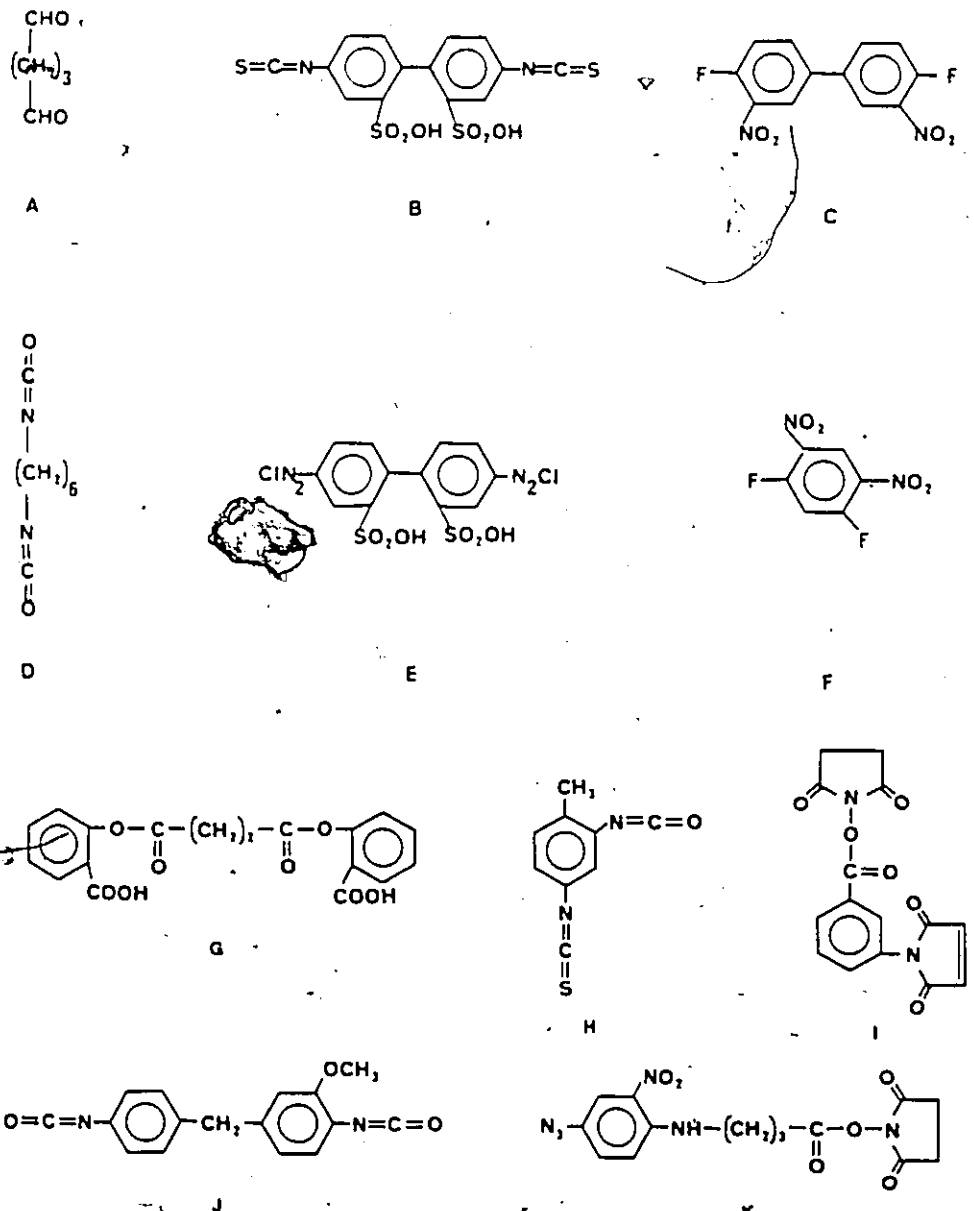
MULTIFUNCTIONAL REAGENTS FOR CROSS-LINKING PROTEINS

Legend

Shown in Figure 1 are multifunctional reagents used for cross-linking proteins. Homogeneous functional group reagents include (A) glutaraldehyde; (B) 4,4'-dithiocyanatobiphenyl-2,2-disulfonic acid; (C) 4,4'-difluouro-3,3'dinitro-biphenyl; (D) hexamethylenediisocyanate; (E) bisdiazobenzene-2,2'-disulfonic acid; (F) 1,5-difluoro-2,4-dinitrobenzene; and (G) succinyldisalicylate. Heterogeneous functional groups reagents include: (H) toluene-2-isocyanato-4-isothiocyanate; (I) maleimidobenzoyl-N-hydrozysuccinimide ester; (J) 3-methoxydiphenylmethyl-4-4'diisocyanate; and (K) N-succinylimidyl-6(4'-azido-2'-nitrophenylamino) propionate.

Taken without permission from Carr and Bowers (9).

FIGURE 1



derivatization step for a preformed, insoluble polymer (9). In this method, one of the molecule's functional groups forms a covalent link with the support; the other functional group or groups can then be used to bind a protein. This application has been seen in the construction of glucose reactors (CHAPTER III).

D. COVALENT ATTACHMENT TECHNIQUES

Covalent attachment techniques are the methods most commonly employed for the immobilization of enzymes. This method normally involves the reaction of an aqueous solution of enzyme with an activated, functionalized water-insoluble support to form a covalent bond between the surface of the matrix and a functional group on the enzyme. Normally, the activation reaction is designed to make the functional groups on the support strongly electrophilic. This method although often tedious, provides an immobile enzyme that is firmly bound to its polymeric support. A range of polymers and chemical coupling procedures which are used are discussed below.

1. Support Considerations

A large range of support materials are available for covalent-binding. Factors which influence the selection of a particular matrix depend on the capacity of the carrier to bind protein, the surface charge and hydrophilicity, the

ease of activation and the interaction of the support with the analyte or sample matrix (8). The mechanical and chemical stability of the support are also important particularly in applications using packed-bed reactors. Supports rich in hydrophobic groups (e.g. aromatic residues) generally give rise to preparations of low protein content and low enzyme activity and often exhibit low stability, while supports rich in hydrophilic groups bind on the average larger amounts of protein and retain a higher proportion of its activity and are more stable (9).

(a) Polysaccharides

The polysaccharides, agarose and cellulose and their derivatives, are commercially available matrices that are widely applicable for the covalent coupling of enzymes. These supports, which are often used in bead form, are porous and possess various degrees of cross-linking which give rise to good binding capacity (9). The sugar residues in these polymers contain hydroxyl groups which are ideal functional groups for participation in covalent bonds. The hydroxyl groups also form hydrogen bonds with water to create a hydrophilic environment in the matrix. However, polysaccharide supports are susceptible to microbial/fungal disintegration, and organic solvents can cause shrinkage of the gels (14). In addition, the highly porous structure of Sephadex, agarose, and other weakly cross-linked polymers is

easily deformed under conditions of flow. Flow rates of greater than few tenths of a mL/min/cm^2 cause compaction of the bed into an impermeable plug and cessation of flow (9).

(b) Vinyl Polymers

Vinyl polymers, such as those based on polystyrene have been used more in immunoassays as beads or tubes. The reason for this is that the nonporous matrix does not have the requisite surface area needed to produce an immobilized enzyme catalyst with adequate catalytic activity per volume (17). The inherent hydrophobicity of polystyrene and related polymers containing high concentrations of aromatic groups could be minimized to some extent by their "dilution" with a hydrophilic component. Polyacrylamide, on the other hand, is a hydrophilic matrix and has been used in the immobilization of enzymes by entrapment. In addition, this matrix is porous and the pore size can be controlled by altering the amount of cross-linking agent used in the gel polymerization reaction.

(c) Polyamides

The polyamides i.e., nylons, have become quite widely used, particularly among those using immobilized-enzyme tubes with continuous-flow analyzers. These supports generally require a much different mode of activation and possess different mechanical characteristics, compared to the

polysaccharides. Nylons, which are commercially available in a number of forms, e.g., membranes, powders, tubes, hollow-fibers are mechanically strong and non-biodegradable.

The nylons of shorter methylene chains e.g. nylon-6 and nylon-6,6 are relatively hydrophilic and thus suitable for enzyme immobilization. Nylon suffers from chemical inertness of the polyamide backbone leaving only the terminal carboxyls and amines as possible reactive functional groups. To increase the binding capacity of nylon, controlled cleavage of the amide bond to increase the number of amines and carboxyl groups has been employed (12). However, this may compromise the mechanical strength of the support. Alternatively, reactive centers may be introduced via O- and N-alkylation of the backbone peptide bonds (see Alkylation, later in this Chapter).

(d) Inorganic Supports

Inorganic supports e.g. common glass, silica and alumina, normally sold in bead form are strong, and durable and resistant to microbial disintegration or solvent distortion. Inorganic supports can be prepared in most pore diameters (30 - 2000Å) and particle sizes (18). Such highly porous structures are nearly ideal for packed-bed reactor applications.

In the analysis of plasma or whole blood, glass is disadvantageous due to the catalysis of the coagulation

reactions by glass surfaces. Moreover, strong interactions between proteins and silica causes denaturation of the enzyme. Controlled-pore glass and controlled pore silica are expensive and best suited for gel permeation chromatography (17). In addition, many inorganic-matrix materials are heterogeneous and contain many metal ions and have a physically and chemically ill-defined surface and are less hydrophilic than the polysaccharide materials.

2. Protein Considerations

The type of functional group on the protein through which the covalent bond with the support is to be formed should obviously be non-essential for catalytic activity; moreover, binding reactions should be carried out under relatively mild conditions and preferentially in aqueous media. Such reactions should exhibit, under ideal conditions, relatively high specificity toward one type of functional group on the protein and minimal side reactions with other functional groups or with the aqueous medium.

The protein functional groups that can be utilized in principle for the covalent binding of enzymes to polymeric supports include:

(1) amino groups, the ϵ-amino groups of lysine and the α-NH_2 groups of the N-termini;

(2) carboxyl groups, (β- and γ-carboxyl groups of aspartic and glutamic acid and terminal α-'s;

(3) phenol rings of tyrosine;

(4) sulphydryl groups of cysteine;

(5) hydroxyl groups of serine, threonine and tyrosine;

(6) imidazole groups of histidine;

(7) indole groups of tryptophan.

In practice, most of the common covalent coupling reactions involve amino groups, carboxyls or the aromatic rings of tyrosine and histidine. In addition, immobilization of glycoenzymes has also been achieved through the covalent attachment of the carbohydrate moiety of the enzyme to the support, which will be discussed later in this Chapter.

3. Support Activation

(a) Cyanogen Bromide Activation

The most prevalent method for activation of polysaccharides for protein immobilization is the use of cyanogen bromide (19). Enzymes with free amino groups can be attached covalently to the activated polymer at high pH (10-12.5) resulting in the formation of three different types of structures (19): N-substituted carbamates, N-substituted imidocarbonates and N-substituted isoureas (Figure 2), the latter being the major reaction product.

The activating agent and its by-products are highly toxic (17), and this method is characterized by a small and constant leakage of bound ligand, due to the electrophilic nature of the isourea bond formed. Moreover, vigorous

FIGURE 2

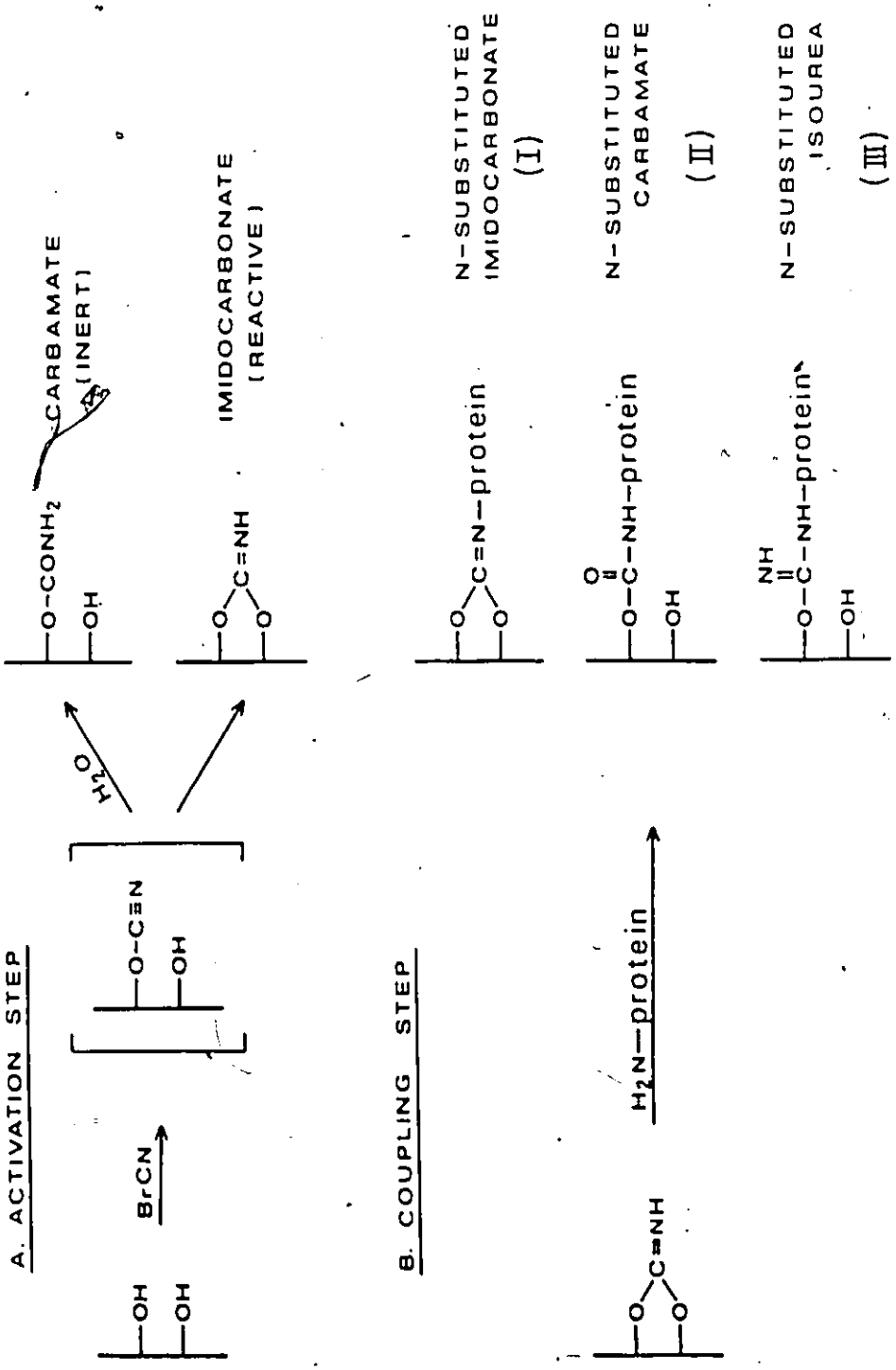
CYANOGEN ACTIVATION PRODUCTS

Legend

Shown in Figure 2 is a scheme for the coupling of proteins via cyanogen bromide activation of polysaccharides. The coupling is thought to proceed through an unstable cyanate and result in the formation of three different types of structures. The substituted isourea structure is most probably the major reaction product (19).

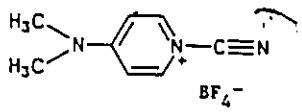
Figure taken without permission from Axen and Ernback (19).

FIGURE 2

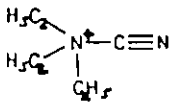

A. ACTIVATION STEP
BrCN
CARBAMATE (INERT)
IMIDOCARBONATE (REACTIVE)
B. COUPLING STEP
N-SUBSTITUTED IMIDOCARBONATE (I)
N-SUBSTITUTED CARBAMATE (II)
N-SUBSTITUTED ISOUREA (III)
protein

activation gives rise to cross-linking of the gel, which, while it improves the mechanical strength, decreases the pore size. Despite its widespread use, cyanogen bromide activation is not the method of choice for most applications for the above reasons.

The same product of cyanogen bromide activation may be achieved with cyanogen-bromide analogs, namely, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (I), and N-cyano-triethylammonium (II) (19,20).



(I)



(II)

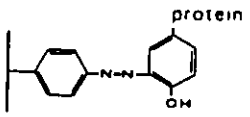
These analogs reduce much of the danger associated with cyanogen bromide while furnishing supports with higher activation densities (20).

In general, polyhydroxylic matrices, e.g., agarose, cellulose and dextran are activated via changing the pre-existing unreactive hydroxyl group into a reactive function, which may then couple to protein nucleophiles, chiefly via the lysyl side chain amine.

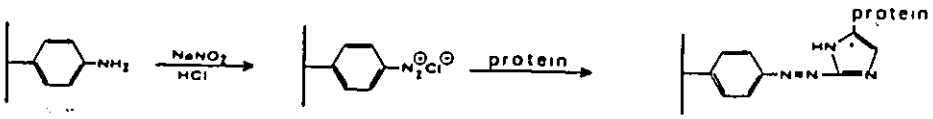
(b) Diazotization

Azo coupling of proteins can be effected by polymers containing aryldiazonium functional groups. The electrophilic aryldiazonium ion attacks mainly activated aromatic rings, such as phenols (tyrosine) or imidazole (histidine)

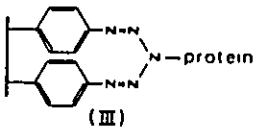
to form the corresponding azo derivatives.



(I)



(II)



(III)

The specificity of azo coupling is rather broad; diazotizating reagents have been shown to attack several other groups in proteins (17).

Aryldiazonium groups, because of their essentially hydrophobic character have adsorptive properties of their own and may tend to attack preferentially hydrophobic, tyrosine rich regions in a protein; such site-directed specificity could cause irreversible damage if key regions of the enzyme are affected. In the case of highly hydrophilic support materials, such as polyelectrolyte diazotized resins, or highly solvated polysaccharide and glass surfaces with diazonium side chains, the site-directed specificity of aryldiazonium groups is mitigated to a large extent.

(c) Alkylation

Alkylation is an activation method widely used with nylon tubing. The earlier methods for the activation of nylon involved hydrolysis of the peptide bonds of the matrix

with acid to liberate amino and carboxyl groups, or cleavage of the nylon with N,N-dimethyl-1-3-diaminopropane to obtain free amino and amide groups (21). This method along with other nylon activation schemes is shown in Figure 3. The backbone can then be resealed by using a four-component mixture of an amine, a carboxyl group, isocyanide, and an aldehyde to form a N-substituted amide (22). The amine and carboxyl groups formed from the hydrolysis can be reconnected by the condensation with acetaldehyde and 1,6-diisocyanohexane. However these procedures which involve hydrolysis of the nylon backbone may compromise the mechanical strength of the tubing.

Activation of nylon by O-alkylation of the secondary amine, has been accomplished by incubating the tube with dimethyl sulphate at 100°C (23). The reactive secondary imidate can also be introduced with triethyloxonium tetrafluoroborate at room temperature (24). Imidoesters are readily attacked by nucleophiles and react selectively with α- and ϵ- amino groups of proteins to form amidines. The latter method, which is used in the present study, is more favorable since the reagents are less caustic, and the reaction is effected at room temperature. A summary of the nylon activation schemes is shown in Figure 3.

(d) Carbodiimide Activation

The most general methods for the activation of carboxyl

FIGURE 3

NYLON ACTIVATION SCHEMES

Legend

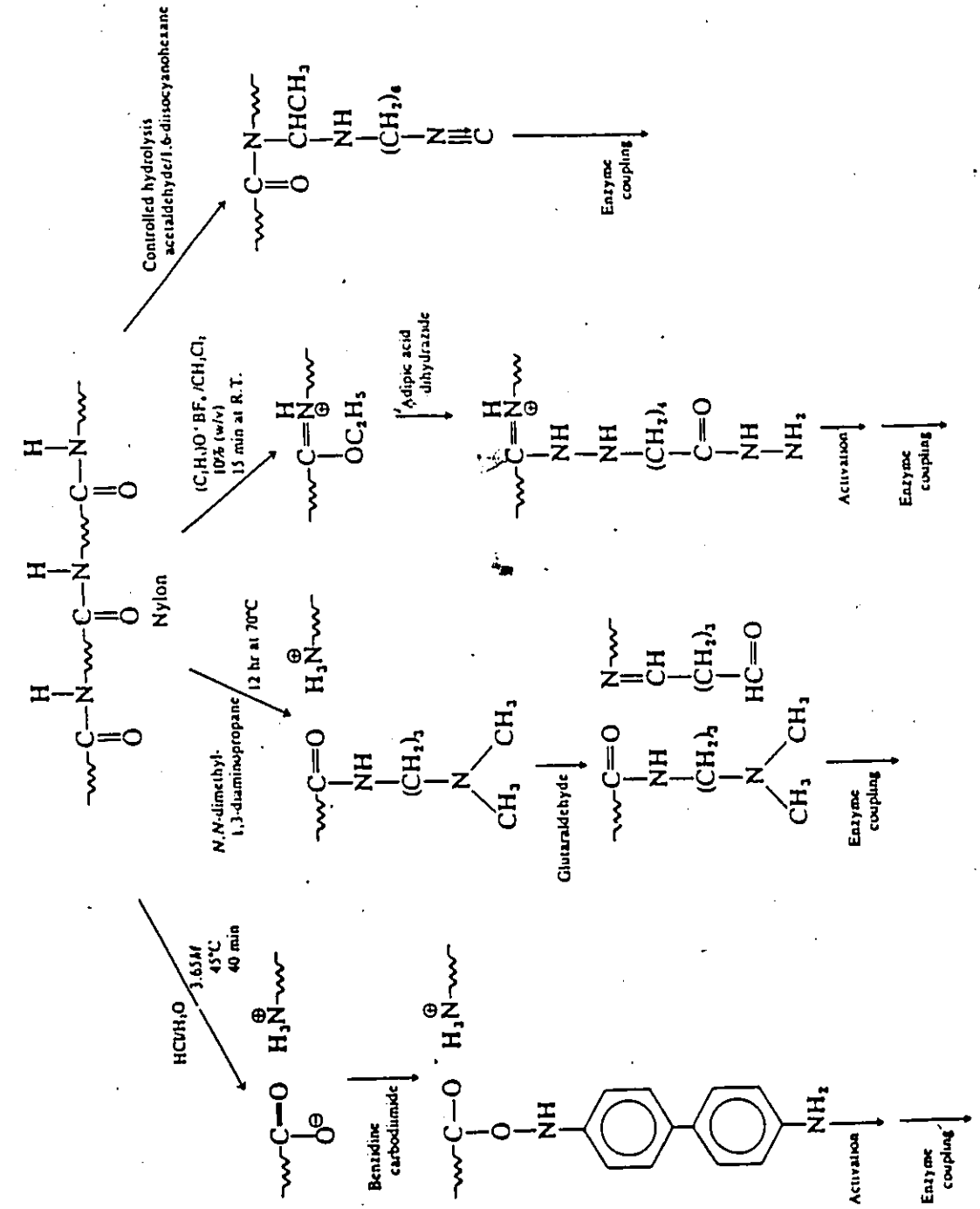
The activation of nylon support by various schemes is shown in Figure 3.

Free carboxyl and amine groups liberated by hydrolysis can be activated with carbodiimide or N,N-dimethyl-1,3-diamopropane. Alternatively, these groups can be resealed with acetaldehye/1,6-diisocyanohexane.

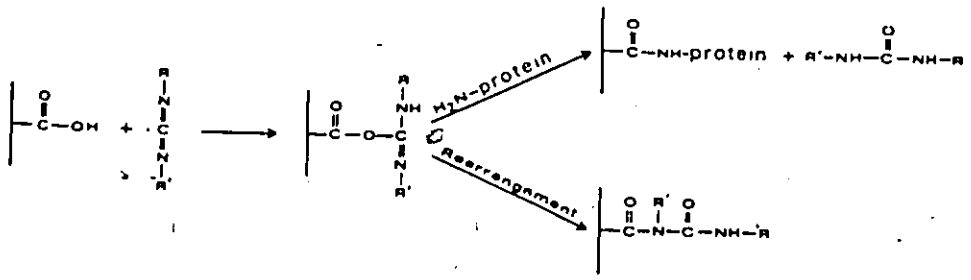
The activation by O-alkylation with triethyloxonium tetrafluoroborate in dichloromethane is illustrated. The imidate salt of nylon can be reacted directly with the enzyme or may be derivatized with acid hydrazides to produce a hydrazide substituted support.

Figure taken without permission from Carr and Bowers (9).

FIGURE 3


Nylon
Controlled hydrolysis
acetaldehyde/1,6-diisocyanohexane
Enzyme coupling
(C2H5)3O+BF4−/CH2Cl2
10% (w/v)
15 min at R.T.
Adipic acid dihydrazide
Activation
Enzyme coupling
N,N-dimethyl-1,3-diaminopropane
12 hr at 70°C
Glutaraldehyde
Enzyme coupling
HCHO
3.65M
45°C
40 min
Benzidine carbodiimide
Activation
Enzyme coupling

groups involve the use of water-soluble carbodiimides and similar reagents. Carbodiimides react with carboxyl groups at slightly acidic pH values (pH 4.75-5) to give O-acyl isourea derivatives.



These highly reactive intermediates can rearrange to an acyl urea or condense with amines to yield the corresponding amides.

(e) Immobilization of Glycoenzymes

Up to 1974, all the chemical techniques of immobilization involved only the modification of the amino acid residues of an enzyme, even though the molecule may have contained other functional groups that could have been employed. Although the exact role of the carbonydrate moiety in glycoenzymes is still not resolved, evidence (25) in most cases, argues against their involvement in catalysis. The principle method is outlined in Figure 4, and consists of activating the glycoenzyme via periodate oxidation followed by contacting the modified "aldehydic" enzyme with an amino-containing water insoluble polymer. This results in active enzyme-polymer conjugates through imine or hydrazone link-

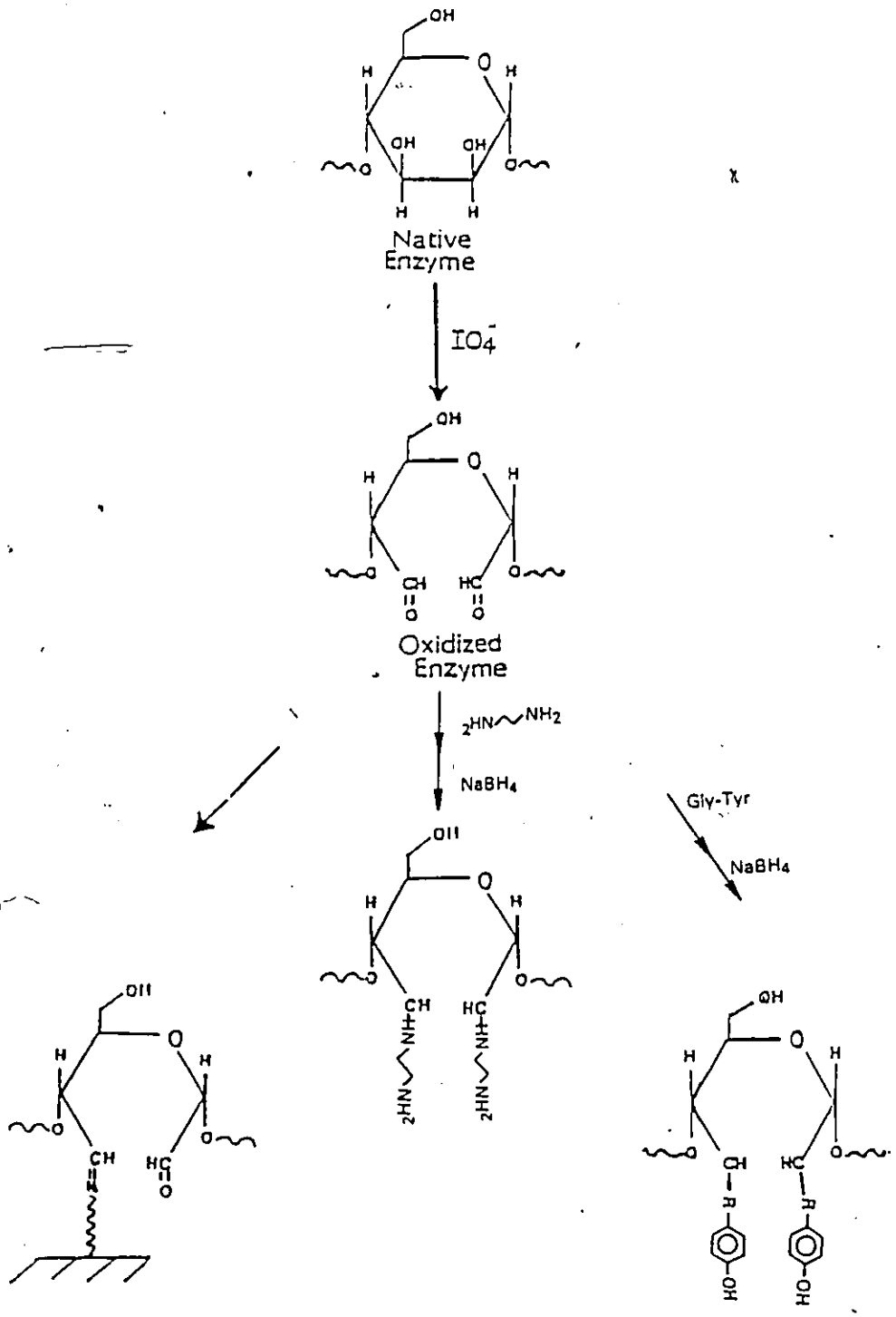
FIGURE 4

IMMOBILIZATION OF GLYCOENZYMES

Legend

Oxidation of the carbohydrate moiety of glycoenzymes with periodate to yield aldehydic groups is shown. The enzyme can then be immobilized through Schiff base formation between the oxidized enzyme and an amino substituted support. Alternatively, stable enzyme derivatives can be formed by the attachment of ethylenediamine or glycyl tyrosine to the carbohydrate chains.

FIGURE 4


Native Enzyme
IO_4^-
Oxidized Enzyme
$H_2N{\sim}NH_2$
$NaBH_4$
Gly-Tyr
$NaBH_4$

ages. In this chemical method, bonding is dependent on the ability to oxidize carbohydrate residues of the glycoenzyme without losing activity. This method is relatively simple and the main advantage is the immobilization via catalytically non-essential residues of the enzyme. Immobilization of the glycoenzymes glucose oxidase and peroxidase through their carbohydrate side chains has been achieved in our laboratory (26).

CHAPTER III

APPLICATIONS IN THE CLINICAL CHEMISTRY LABORATORY

A. INTRODUCTION

Practical applications of immobilized enzyme technology has been realized in the treatment of industrial wastes, the production of food and drugs, in clinical analysis and as therapeutic agents. Industrial applications of immobilized biocatalysts has been seen in the form of the removal of lactose from milk by immobilized lactase, or in the form of glucose isomerase reactors for the industrial scale production of high fructose syrups (1). Therapeutic applications of immobilized enzyme has been seen in the removal of undesirable products from the blood using an extracorporeal device (27), or in enzyme replacement therapy usually in the form of an encapsulated enzyme (28). As indicated earlier, immobilized enzymes are suitable for incorporation into instruments designed for a continuous re-use operation. In particular, immobilized enzymes have been featured in the development of several analytical biosensor devices, namely the enzyme electrode and the enzyme reactor. In addition, a number of immobilization methods have been described for the glucose oxidase or dehydrogenase and urease enzymes. This is probably because of the stability of these enzymes, the availability and the clinical application of these enzymes

for the assay of glucose and urea, respectively. For these reasons, the review will focus primarily on the immobilization technology for the determination of these analytes.

B. ENZYME ELECTRODES

Enzyme electrodes are probes capable of generating a electrical potential as a result of a reaction catalyzed by an enzyme that is immobilized on the surface of an electrochemical sensor (29). The immobilization is generally achieved by physical entrapment, chemical cross-linking or by covalent coupling of the enzyme onto the electrode surface, with the latter the most suitable for the preparation of stable electrodes. The enzyme electrode (Figure 5), which may be envisaged as a self-contained analytical biosensor, consists of an enzyme layer held in close proximity to a transducer (which might be a potentiometric or amperometric indicator electrode), a reference electrode and a circuit for measuring either the potential difference between the 2 electrodes or the current which flows between them. After 30 seconds to 2 minutes (seldom more than 10 minutes), a steady state potential or current related to the analyte concentration is reached. The potentiometric sensor could be any one of a number of ion-selective sensors. Platinum amperometric sensors, which can reduce oxygen or oxidize peroxide have been employed for enzyme electrodes. The analytically useful ranges of these probes is from 0.1

FIGURE 5

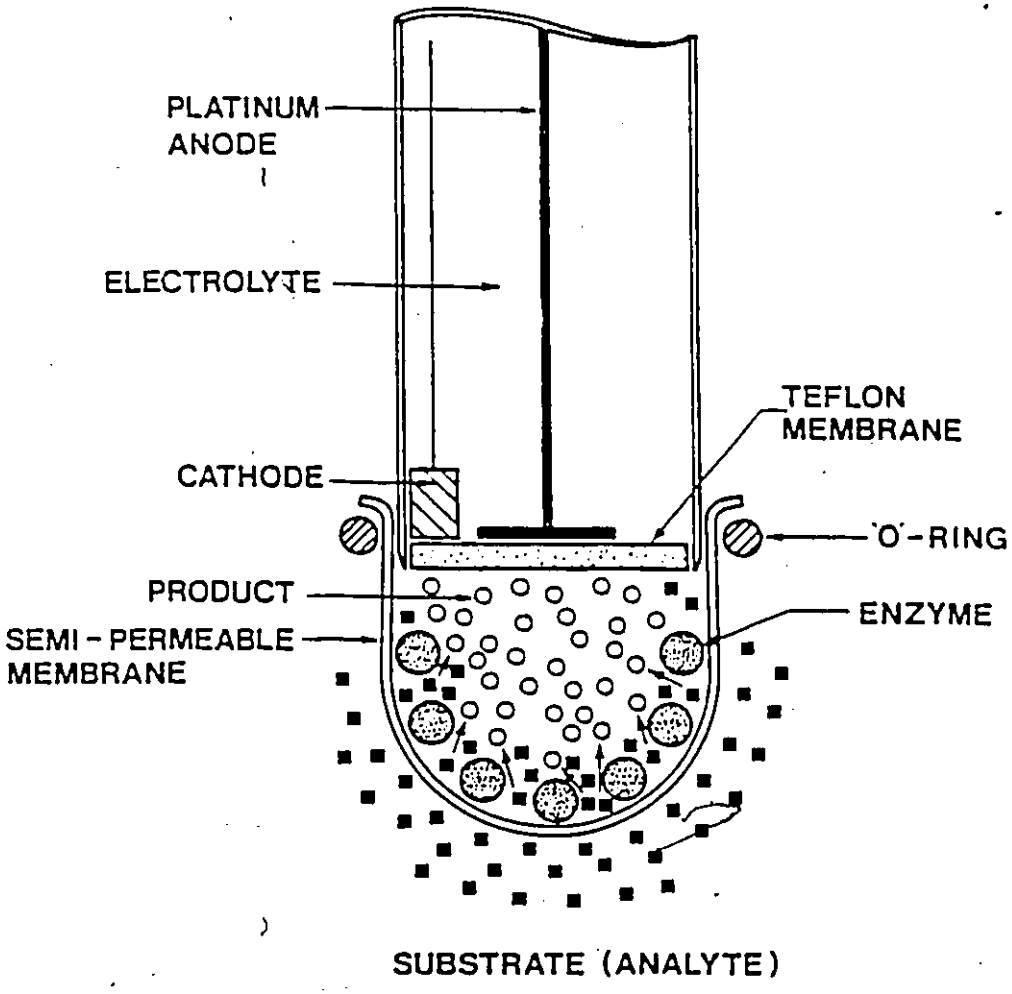
DIAGRAM OF A SIMPLE ENZYME ELECTRODE

Legend

Diagram of a simple enzyme electrode biosensor device, combining an electrochemical electrode and an enzyme immobilized onto a semi-permeable membrane.

Figure taken without permission from Bickerstaff (8).

FIGURE 5


PLATINUM
ANODE
ELECTROLYTE
TEFLON
MEMBRANE
CATHODE
O-RING
PRODUCT
ENZYME
SEMI-PERMEABLE
MEMBRANE
SUBSTRATE (ANALYTE)

to 10 μM (30).

1. Glucose Electrodes

A survey of the enzyme-based electrode literature shows that a vast majority of biosensors have been devoted to glucose monitoring. The first attempt to construct immobilized enzyme electrodes for blood glucose determination (31) was based on the following reaction:

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{GO} \text{D-glucono-}\delta\text{-lactone} + H_2O_2 \quad (1)$$

$$\text{D-Glucono-}\delta\text{-lactone} + H_2O \longrightarrow \text{D-gluconic acid} \quad (2)$$

By entrapping glucose oxidase (GO) in a polyacrylamide gel and coating a platinum electrode, oxygen depletion could be measured which is directly proportional to the glucose concentration (31). Since then, several glucose probes dealing with oxygen depletion have been reported, however, these have limitations in whole blood and plasma due to the variability of oxygen tension.

The amperometric monitoring of liberated hydrogen peroxide (eqn. 1) has been preferentially investigated since peroxide is readily oxidized at platinum electrodes. Such an electrode has been constructed by Lubrano and Guilbault (32), where glucose oxidase which was immobilized by cross-linking with bovine serum albumin (BSA) and glutaraldehyde was used to cover a platinum glass layer. The current produced is proportional to the glucose concentration. Reading times were less than 12 s, and the electrode was stable for over 1 year at room temperature. Glucose oxidase

has been immobilized by a number of techniques: (i) by crosslinking with BSA and glutaraldehyde (32); (ii) covalent linkage on nylon mesh (33); or (iii) covalent attachment on to the electrode surface (34,35).

Monitoring of blood glucose has also been achieved with bienzyme electrodes using glucose oxidase-peroxidase (POD), and a redox mediator in the following reactions:

$$\text{Glucose} + O_2 \xrightarrow{GO} \delta\text{-gluconolactone} + H_2O_2 \quad (3)$$

$$H_2O_2 + 2[Fe(CN)_6]^{4-} + 2H^+ \xrightarrow{POD} 2[Fe(CN)_6]^{3-} + 2H_2O \quad (4)$$

The hexacyanoferrate (III) formed is reduced at a glassy electrode (36).

Other enzymatic systems have also been tested; for example, glucose has been determined by using glucose dehydrogenase immobilized at a platinum electrode (37). By using an electron acceptor, i.e., 2,6-dichlorophenol indophenol, glucose can be assayed despite a fairly long electrode response time (37).

2. Urea Electrodes

Blood urea determination is one of the most frequently required clinical tests in diagnosis, because urea concentrations are influenced by not only diet and malnutrition, but also by renal failure and other infective or degenerative causes of impaired glomerular filtration. Like glucose, urea, has been the subject of numerous investigations, mainly with immobilized potentiometric gas-selective

electrodes which monitor the ammonia formation in the reaction:

$$(NH_2)_2CO + 2H_2O + H^+ \xrightarrow{\text{urease}} HCO_3^- + 2NH_4^+ \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} NH_3 \quad (5)$$

The first stable enzyme electrode was based on the combination of urease trapped in polyacrylamide matrix placed over a cation-selective electrode which responded to NH_4^+ (38). Because sodium and potassium interfered with the ammonium sensor, attention has been directed to the preparation of interference-free direct reading electrodes (39). By immobilizing a thin layer of urease via cross-linking with BSA and glutaraldehyde, such an electrode has been developed by Mascini and Guilbault (39) and was stable for up to one month.

Vincke et al. (40), also using a NH_3 gas electrode have immobilized urease onto a cellulose acetate membrane with glutaraldehyde or by entrapment in a gel (agar). Comparison of these electrodes with a spectrophotometric procedure gave good correlation and could be used over the range of 0.5 μM - 10 mM for 200 to 1000 assays.

Begum and Mottola (41) have described an urea enzyme electrode which incorporated nylon shavings that had been alkylated. Finally, urea has been determined in diluted plasma (42) and serum samples (43) by covering the tip of a glass pH electrodes with a thin layer of immobilized urease physically entrapped in a polymer gel.

3. Other Electrodes

The scope of application of enzyme electrodes is in principle is very broad: any enzyme which generates hydrogen peroxide or consumes oxygen could be the basis for an enzyme probe using amperometric detection. For example, determination of oxalate (44), galactose (45), salicylate (44), and cholesterol (46), with their respective oxidases or hydroxylases, have been based on the following reactions:

$$\text{Oxalic acid} + O_2 \xrightarrow{\text{oxalate oxidase}} 2CO_2 + H_2O_2 \quad (6)$$

$$\text{D-Galactose} + O_2 \xrightarrow{\text{galactose oxidase}} \text{D-galacto-hexodialdose} + H_2O_2 \quad (7)$$

$$\text{Salicylate} + \text{NADH} + H^+ + O_2 \xrightarrow{\text{salicylate hydroxylase}} \text{o-dihydroxybenzene} + \text{NAD}^+ + H_2O + CO_2 \quad (8)$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{4-cholesten-3-one} + H_2O_2 \quad (9)$$

Alternatively, both oxalate and salicylate have been determined potentiometrically (47) using a carbon dioxide probe. In addition, a uricase electrode has been developed consisting of a platinum disk in contact with a layer of glutaraldehyde gel in which the uricase is immobilized (48). The urate is determined as follows:

$$\text{Urate} + 1/2O_2 \xrightarrow{\text{uricase + catalase}} \text{allantoin} + CO_2 \quad (10)$$

Table I summarizes the applications of enzyme electrodes in clinical chemistry.

TABLE I

EXAMPLES OF THE APPLICATIONS OF ENZYME ELECTRODES IN CLINICAL CHEMISTRY

Analyte	Enzyme	Sensor	Ref.
Cholesterol	Cholesterol oxidase	O_2	46
Galactose	Galactose oxidase	O_2	45
Glucose	Glucose oxidase	O_2	31
		H_2O_2	32-35
	Glucose oxidase, peroxidase	Hexacyano-ferrate(III)	36
	Glucose dehydrogenase	O_2	37
Oxalic acid	Oxalate oxidase	O_2	44
		CO_2	47
Salicylate	Salicylate hydroxylase	O_2	44
Urea	Urease	NH_4^+	38-43
Uric acid	Uricase, Catalase	CO_2	48

C. IMMOBILIZED ENZYME REACTORS (IMER'S)

One format for automatic analyzers in Clinical Chemistry, operates on a continuous-flow of samples and reagents through a system of channels to mixing chambers and then on to detecting devices for estimation of the reaction. The most appropriate format of the immobilized enzyme for such systems is the enzyme reactor (IMER). In practical terms, the enzyme reactor would replace the mixing chamber in the conventional automatic analyzer. A typical system is outlined in Figure 6. The samples are introduced into an air segmented liquid stream separated by a wash liquid. The liquid stream carrying the samples passes through the enzyme reactor which acts as a mixing coil. The air-segmented liquid stream passes through a flow cell and a signal proportional to the concentration of the analyte is read.

Two types of enzyme reactors have been investigated for use in continuous-flow analysis - the tubular enzyme (coil) reactor and the packed bed (column) reactor. In the packed bed reactor, the enzyme is immobilized for e.g. onto porous beads or within fibers, and then the immobilized enzyme is packed into a glass or plastic tube to form a small enzyme reactor column. In the tubular system, the enzyme is attached onto the inner surface of a length of tubing. As will be shown through many examples, nylon tubing is very suitable and has been incorporated into automated analyzers.

The following section will review the applications of

FIGURE 6

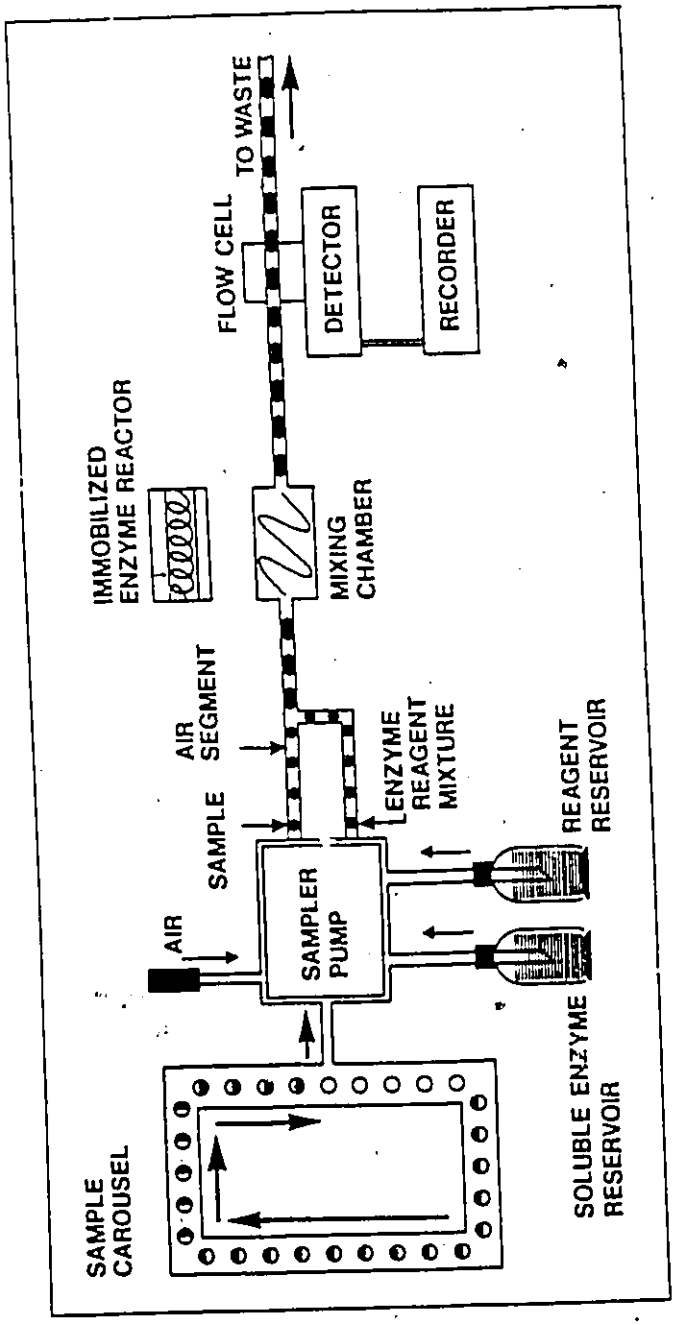
OUTLINE OF A FLOW SYSTEM FOR AUTOMATED ANALYSIS USING AN IMMOBILIZED ENZYME REACTOR

Legend

Shown in Figure 6 is a schematic illustration of a continuous-flow analyzer with segmented flow. The samples are introduced into an air segmented liquid stream separated by a wash liquid. The liquid stream carrying the samples passes through the enzyme reactor which acts as a mixing coil. The air-segmented liquid stream passes through a flow cell and a signal proportional to the concentration of the analyte is read.

Figure taken without permission from Bickerstaff (8).

FIGURE 6

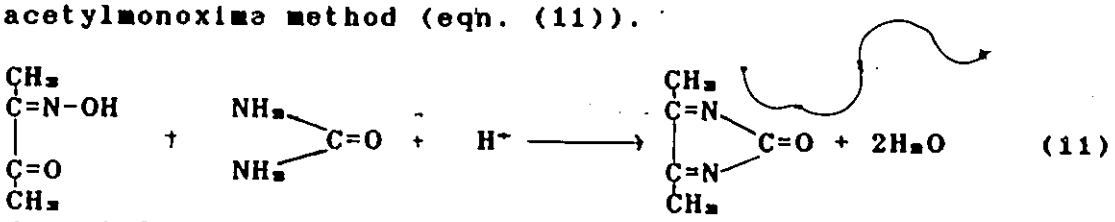

TO WASTE
FLOW CELL
DETECTOR
RECORDER
IMMOBILIZED ENZYME REACTOR
MIXING CHAMBER
AIR SEGMENT
SAMPLE
ENZYME REAGENT MIXTURE
REAGENT RESERVOIR
AIR
SAMPLER PUMP
SAMPLE CAROUSEL
SOLUBLE ENZYME RESERVOIR

IMER's in the clinical chemistry laboratory, once again with emphasis on the determination of the analytes glucose and urea.

1. Urea Determination

The first successful analytical application of an immobilized-enzyme preparation was the determination of urea in serum and urine using immobilized urease and a colorimeter (49) (eqn. (5)). Although a good correlation was reported between the immobilized enzyme reactor and the Berthelot method, the primary goal of the paper was the characterization of the bound urease. As a result, analytical details were rather sparse. In addition, the analysis time using this method was 1 hour per sample. The enzyme determination of urea by a technique that is more economical and relatively easy was first demonstrated by Sundaram and Hornby (50). Urease was immobilized onto a 2-m length of nylon-tube support by linking through glutaraldehyde and the urea assayed in a continuous flow system. Since then, the same principle has been further developed, with urease reactors made by the cross-linking method of Sundaram and Hornby (50) or by direct coupling to nylon alkylated with dimethyl sulphate (51). In these procedures, the urease reactor was integrated in the flow system of a Technicon AutoAnalyzer I and the urea measured by assaying the effluent for ammonia by the Berthelot reaction or by the di-

acetylmonoxime method (eqn. (11)).



diacetyl urea diazine derivative (yellow) (11)

Routine determinations were done at a rate of 60 samples per hour and the reactors were stable for 4 months and 2000 tests. The reactors made by direct coupling to alkylated tube lose about 35% activity after 2 weeks of continuous use whereas the reactors made by urease cross-linked to the partially hydrolyzed tube lose about 22% activity in the same time. Subsequently, urease has been immobilized on various lengths of nylon tubing that have attached diamine spacer arms to the alkylated surface to minimize the problem of enzyme multipoint attachment (52,53). The arms are then activated with bifunctional reagents prior to enzyme coupling (53). The immobilized urease obtained had a shelf-life of 8 months and could be used for at least 10,000 urea assays. Tubes of such manufacture are commercially available from Carlo Erba (Italy) in a cartridge housing.

In an attempt to minimize non-specific binding of urease to nylon, new methods of enzyme immobilization have been developed in which the nylon is coated with polyaminated derivatives of starch and dextran (54,55). These derivatives which act as spacer arms were employed to make a more hydrophilic support. The arms are once again activated with glutaraldehyde prior to enzyme coupling. This procedure

however, resulted in lower urease activity than observed with other tube modifications.

2. Glucose Determination

The estimation of glucose in blood serum is one of the most frequent analyses performed in the clinical laboratory. Two enzymatic methods are commercially available for the assay of glucose: the glucose oxidase/peroxidase combination for the oxidation of a chromogen, and the hexokinase/-glucose-6-phosphate dehydrogenase (G6PDH) combination for the reduction of $NAD^+/NADP^+$.

(a) Glucose Oxidase Reactors

In 1966, Updike and Hicks (56) reported a system using polyacrylamide gel-entrapped glucose oxidase (GO) and lactate dehydrogenase (LD) for the determination of glucose and lactate, respectively. However, the major emphasis of the paper was a study of the characteristics and preparation of the immobilized enzyme. Using another entrapment procedure, Campbell and coworkers (57) showed that glucose oxidase could be immobilized between two dialysis membranes. The resultant enzyme-membrane sandwich reactor was employed in the dialyzer unit of a Technicon AutoAnalyzer I at 37°C and found to perform efficiently in the automated analysis of glucose. In another study, (58), using a coiled configuration, immobilized glucose oxidase was incorporated into a

Technicon AutoAnalyzer™ for the assay of glucose. By using a relatively "thick" enzyme coating, only a 30-cm length of nylon was needed and up to 60 samples/hour could be assayed and up to 25,000 assays per tube. In these tubes, however, the immobilized enzyme is not bound covalently to the wall, but is present in an insoluble porous annulus, which adheres to the tube wall. Thus the nylon tubing serves as a cylindrical envelope, rather than a bona fide support for the immobilized enzyme. In addition, this method complicates the reactor kinetics.

Using a procedure of Inman and Hornby (59), Joseph et al., (60) covalently immobilized glucose oxidase onto a 100-cm length of nylon tubing using glutaraldehyde activation and once again showed that the coiled tube obtained could be incorporated into the Technicon AutoAnalyzer™ for analysis of serum glucose. Other investigators (52,53,61) have also shown the use of immobilized glucose oxidase reactors in continuous-flow analyzers in the form of nylon tubing of various lengths. The latter, however, presented a shorter procedure for immobilization on nylon by reducing the immobilization time from ~4.5 hours to 80 minutes by eliminating certain wash procedures, and reducing incubation times on certain modification steps. This resulted in higher apparent activities of the enzyme. More recently, a process using p-benzoquinone in dioxane has been applied for the activation of polyacrylamide beads (62). In this study, an

increase in the stability of immobilized glucose oxidase on this strongly hydrophilic environment was observed. Preliminary experiments show that this method may have applications for the determination of glucose in biological fluids.

In our laboratory, glucose oxidase has been coimmobilized with horseradish peroxidase onto nylon tubings treated with limited amounts of alkylating reagents and long spacer arms (27), and preliminary work has shown that this procedure can be used for assaying glucose in serum and urine.

(b) Dehydrogenase Reactors

(i) Glucose Dehydrogenase Reactors

The determination of plasma glucose has also been achieved using a stirrer containing immobilized glucose dehydrogenase (63). In this procedure, the imido groups on cyanogen bromide activated cellulose were modified by adding ethylene diamine and glutaraldehyde to provide spacing groups. Estimates show that the stirrer could be used for routine analysis for 2 months or about 500 runs. Sundaram et al., (64) have reported a method for immobilizing glucose dehydrogenase on the inside surface of nylon tubes by cross-linking the enzyme to a nylon-polyethyleneimine copolymer by use of glutaraldehyde (65). In this case, a 1-m length of coiled nylon tubing was incorporated into a Technicon AutoAnalyzer™ and retained 80% of its activity after 3500 analyses. A procedure for coupling of glucose dehydrogenase

to glass tubes has been described by Bisse et al.,, (66). The tubes which were activated with transition metal salts such as $TiCl_4$ were formed to a coil and incorporated in a Technicon system. The stability of this preparation was less than those of the nylon reactors.

(ii) Hexokinase/Glucose-6-Phosphate Dehydrogenase Reactors

Glucose analysis making use of immobilized hexokinase-G6PDH was first suggested by Morris et al., (24) for use in automated continuous-flow systems. Coils were prepared by alkylation of the inner walls of small bore nylon tubes with triethyloxonium tetrafluoroborate (TEOTFB) and subsequently reacted with a diamine. The tubes were then activated with glutaraldehyde and the enzymes co-immobilized. This yielded a relatively "thin" enzyme coating on the wall, therefore, tubes as long as 1-m were required in order to achieve acceptable assay sensitivity. Other workers (67), have demonstrated that such tubes could be used on the Technicon SMAC system for the accurate analysis of glucose. In this case, however, tubes 30-cm long were sufficient to achieve the desired sensitivity.

Jablonski and DeLuca (68) have investigated the use of a light generating system for the determination of glucose and glucose-6-phosphate. In this study, luciferase and oxidoreductase were coimmobilized on glass rods using the

method of diazotization to activate the carrier and the analytes determined according to the following scheme.

$$NAD(P)^+ + \text{glucose} \xrightarrow{\text{GDPDH}} NAD(P)H \quad (12)$$

$$NAD(P)H + FMN + H^+ \xrightarrow{\text{oxidoreductase}} NAD(P)^+ + FMNH_2 \quad (13)$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + h\nu \quad (14)$$

The same method has been applied to obtain other coimmobilized multienzyme systems (69,70). Bacterial luciferase and oxidoreductase were coimmobilized onto alkylamino glass beads with glucose-6-phosphate, lactate, and malate dehydrogenase to quantify their substrates. Kricka et al., (71) have developed very sensitive assays for glucose, glucose-6-phosphate and 6-phosphogluconate using mutienzymatic systems coimmobilized with a bacterial system on CNBr-Sepharose packed into small flow cells.

The immobilized enzymes of the bacterial bioluminescent system are fairly promising primarily for assaying NAD(P)H, and $NAD(P)^+$ NAD(P)H-dependent enzymes and their substrates. Schoelmerich et al., (72) have developed an assay for bile acids using coimmobilized 3- -hydroxysteroid dehydrogenase and bacterial luciferase. The enzymes which were immobilized on CNBr-Sepharose, provided a convenient and sensitive assay for the determination of bile acids in blood serum when compared to chromatographic and radioimmunological assays. Similarly, very sensitive assays have been developed for various hormones (73,74) using coimmobilized luciferase, oxidoreductase and the substrate's dehydrogenase. Compared

to the arylamine glass bead immobilized enzymes, the Sepharose-immobilized preparations had a much higher activity.

3. Other Reactors

Analytical methods for the continuous-flow analysis of uric acid, which is essential in the diagnosis of gout, using immobilized uricase (urate oxidase) have been examine by Sundaram et al., (75), Chirillo et al., (52) and Salleh et al., (76). Whereas Sundaram (75) and Salleh (76) used nylon coils of 1-2 m in length, Chirillo (52) obtained bound uricase coils with sufficiently high activity so that adequate sensitivity could be achieved with tubes less than 30-cm long. Results of uric acid analysis by use of immobilized uricase with peroxidase gave good correlation with the soluble enzyme system. Similarly, Thomasson et al., (77) have evaluated bound uricase on nylon for the determination of uric acid in urine. Once again, good correlation was observed with established methods. More recently (78), uricase has been immobilized on protamine bound to glass beads packed in a column, and uric acid determined spectrophotometrically.

In addition, oxalate has been determined using oxalate oxidase (eqn. (6)) immobilized onto nylon supports (26,79). Similarly, pyruvate and lactate have been determined in serum with the use of lactate dehydrogenase (LD) and alanine aminotransferase (ALT) coimmobilized on nylon tubes

(80), according to the equations:

$$\text{Lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} \quad (15)$$

$$\text{Pyruvate} + \text{glutamate} \xrightarrow{\text{ALT}} \text{2-oxoglutamate} + \text{alanine} \quad (16)$$

Lactate and pyruvate have also been determined by use of lactate dehydrogenase immobilized in microparticles of polyacrylamide (81). Table II summarizes the applications of enzyme reactors in clinical chemistry.

D. OTHER APPLICATIONS

Another application of immobilized enzyme technology has been in the form of an enzyme thermistor, which is a simplified flow calorimeter designed for routine analysis and is based on the use of immobilized enzymes. Using about a 50-uL sample volume, the heat evolved in the enzymatic reaction (generally at least 20 kJ/mol), (82) can be utilized to determine calorimetrically the amount of substrate reacted. A limitation with this application is that all enthalpy changes of the system are registered without discrimination. An advantage however, is that a process can be followed irrespective of the physical properties (e.g. turbidity) of the reaction solution. This application is not widely used in clinical chemistry because of the high cost and the need to have thermally regulated instrumentation. Nevertheless, enzyme thermistors have been developed for the assay of glucose (83) and urea (84). For these analytes, the hexokinase and urease were immobilized on controlled poros-

TABLE II

EXAMPLES OF THE APPLICATIONS OF IMMOBILIZED ENZYME REACTORS IN CLINICAL CHEMISTRY

Analyte	Enzyme	Matrix	Ref.
Bile acids	3-α-Hydroxysteroid dehydrogenase, luciferase	CNBr-Sepharose	72
Glucose	Glucose oxidase	Dialysis membr.	57
		Glass	83
		Nylon	26, 52, 53 58, 60, 61 85
		Polyacrylamide	56, 62
	Glucose dehydrogenase	Cellulose	63
		Glass tubes	66
		Nylon	64
	Hexokinase, G6(P)DH	Nylon	24, 67
	Hexokinase, G6(P)DH, Luciferase, Oxidoreductase	Glass rods	68
		Nylon	69-71
Oxalate	Oxalate oxidase	Nylon	26, 79
Pyruvate/ lactate	Lactate dehydrogenase, Amino alanine transferase	Nylon	80
		Polyacrylamide	81
Urea	Urease	Glass	84
		Nylon	49-55
Uric acid	Uricase	Glass beads	78
		Nylon	52, 75-77

ity glass by means of glutaraldehyde cross-linking and the immobilized enzymes incorporated into an enthalpimetric flow analyzer. Both methods appeared to be clinically useful showing good correlation with commonly used methods.

Other applications include the diagnostic test strip in which a thin strip of plastic with a small pad, contains the immobilized enzyme(s) and colour reagents. This was first developed to test for glucose in urine using immobilized glucose oxidase and peroxidase (8). Another interesting application has been the development of the enzyme pipette (or impette) where enzymes are immobilized onto nylon tubes by the methods of Sundaram et al., (51) was added to the end of an automatic pipette tip. Solutions of the same volume are sucked into the impette and allowed to react for 5 minutes, expelled, and the products determined by conventional methods (85). This application, although suitable for research laboratories and private physicians clinics, cannot replace the rapid sampling and vast turnover that is needed in hospital settings.

CHAPTER IV

PRESENT STUDY

As mentioned in CHAPTER I, immobilization of an enzyme often leads to a compromise in the activity when compared to its soluble analogue. Multiple bond formation and uneven distribution of cross-linking groups are two explanations of this phenomenon, therefore, it was suspected that the degree of support derivatization may also be important to the recovery of enzyme activity. Derivatization of nylon by alkylation has been achieved using saturating concentrations of TEOTFB, however, this gives rise to a support which is highly activated and may result in multipoint attachment of the enzyme to the support. The supported enzyme in this case would be expected to be less accessible to substrate and cofactor molecules. Strategies for achieving lower functional group density have focused on the use of milder reaction conditions (86), or the use of limiting reagent concentrations (26). By limiting the concentration of activating reagent, coupling of an enzyme through a single point is more likely, with the preferred functional group density in the range of one per 2000Å^2, the approximate cross-sectional area of a protein with Stoke's radius of 25Å, assuming mono-attachment. This amounts to approximately 83 nmol/m^2 of accessible surface area, approximately 4 nmol/m of 1.5-mm diameter tubing (see APPENDIX I for calcu-

lations). The present work was concerned with the development of an analytical method to characterize functional group density on nylon tubing that had been activated with micromolar concentrations of TEOTFB, to ultimately provide a clue to the availability of reactive centres for subsequent modification.

A. APPROACH

As mentioned in CHAPTER II, activation of nylon tubing has been largely achieved through reaction of the carbonyl group of the nylon with the alkylating agent triethyloxonium tetrafluoroborate (TEOTFB). Activation with TEOTFB, which is hygroscopic and subject to hydrolysis, forms an imidate salt on the support which can be allowed to react in non-aqueous conditions with various nucleophiles as a central route for enzyme immobilization (see Figure 4, CHAPTER II).

Previous work in this laboratory has dealt with the evaluation of the structural features of long hydrophilic spacer arms and the subsequent immobilization of peroxidase, glucose oxidase and oxalate oxidase on nylon through derivitization with these arms (26). In addition, previous work focused on the immobilization of horseradish peroxidase on polyacrylamide beads (87), coimmobilization of enzymes on nylon (26), and the analysis of kinetic parameters and stability studies of certain enzyme conjugates suitable for immobilization (88).

The present study began with a "pitting" procedure (89) using a calcium chloride/methanol mixture which removes amorphous nylon, thereby increasing the surface area available for derivatization. The alkylating reagent TEOTFB, is then provided to the nylon support yielding the imidate salt, the target of the investigation. It was envisaged that if the amount of the imidate salt could be quantitated, this would provide an indication of the functional group density on the nylon tubing available for subsequent modification.

A model reaction for this procedure is the hydrolysis of acetimidate esters. Hydrolysis yields amines and esters in acid solution and amides and alcohols at alkaline pH's, both sets of products occurring through a tetrahedral intermediate (Figure 7) (90,91). As the pH is increased, the yield of the ester begins to decrease until the corresponding amide and alcohol are produced in near-quantitative yield around pH 10 (92).

Production of the alcohol, i.e., ethanol, from the hydrolysis of the imidate salt formed by O-alkylation, should be stoichiometric to the amount of TEOTFB activation sites on the nylon. Therefore, one approach to the analysis of such functional group density would be to analyze for ethanol liberated upon intentional hydrolysis of a sample of the activated nylon tubing.

One strategy for the determination of ethanol would be the optimization of a commercial kit using the alcohol

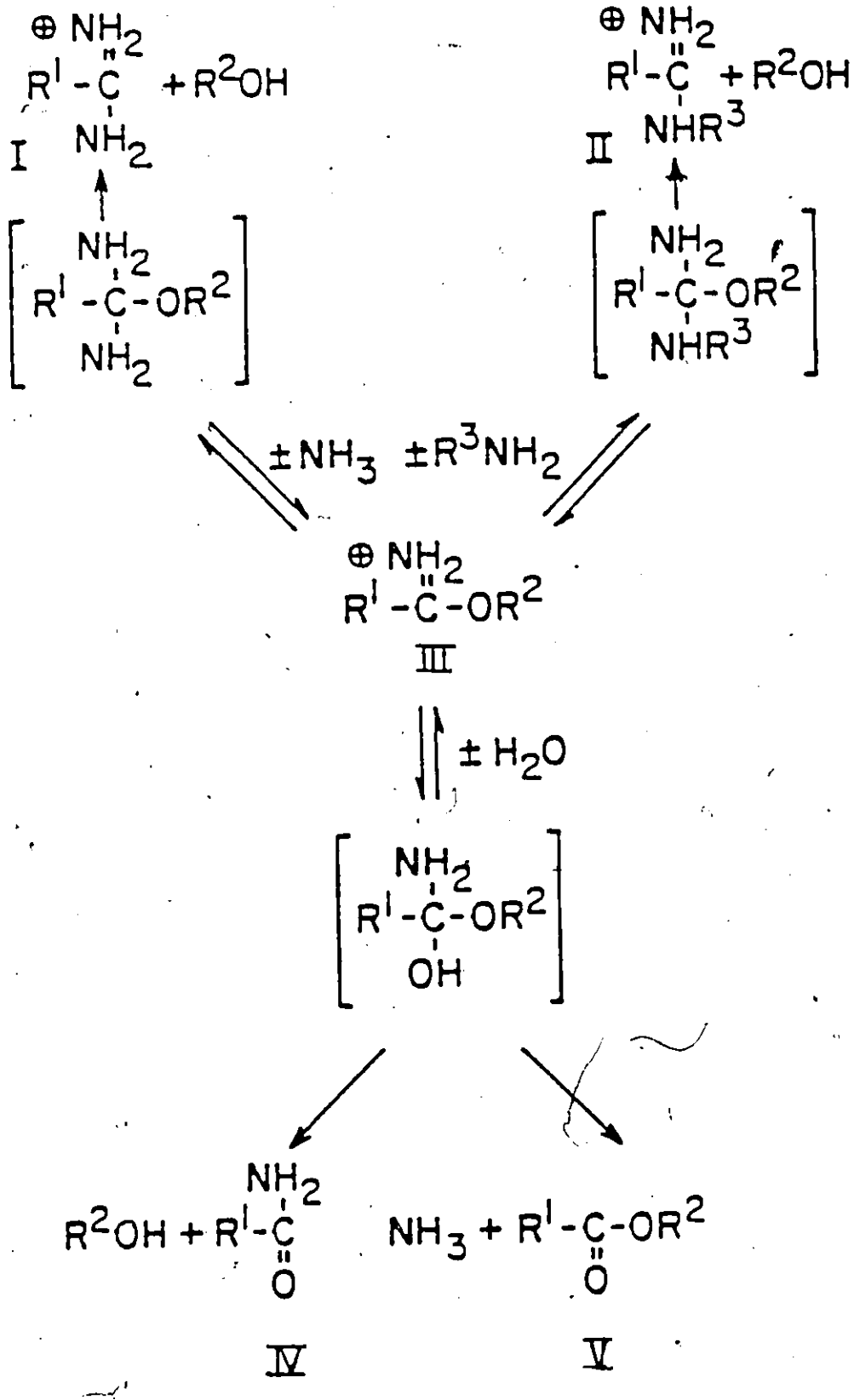
FIGURE 7

REACTIONS OF IMIDOESTERS

Legend

Shown are the principle reactions of imidoesters with water, ammonia and primary amines. The starting reagent is assumed to be structure III. The principal products of hydrolysis are (a) the amide (IV) and the alcohol R"OH or (b) the ester (V) and ammonia. The amidine (II) is formed by the reaction of III with a primary amine.

FIGURE 7


⊕ NH2
R1 - C + R2OH
NH2
I
NH2
R1 - C - OR2
NH2
⊕ NH2
R1 - C + R2OH
NHR3
II
NH2
R1 - C - OR2
NHR3
± NH3
± R3NH2
⊕ NH2
R1 - C - OR2
III
± H2O
NH2
R1 - C - OR2
OH
NH2
R2OH + R1 - C
O
IV
NH3 + R1 - C - OR2
O
V

dehydrogenase/pyridine nucleotide assay. This assay is based on oxidation of the alcohol to the aldehyde and reduction of NAD to NADH which is measured at 340 nm.

$$EtOH + NAD^{+} \xrightarrow{ADH} \text{acetaldehyde} + NADH_{340nm}$$

Another strategy would be the use of a coupled enzyme assay using alcohol oxidase and peroxidase. Action of peroxidase in conjunction with the chromogen system 4-aminoantipyrene (4-AAP) and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS) will produce a red chromogen to be measured at 510 nm (Figure 8). The absorbance at 510 nm is then proportional to the amount of TEOTFB bound to the nylon.

B. MATERIALS

Peroxidase (horseradish) (POD) [donor: hydrogen peroxide oxidoreductase; EC 1.11.1.7] Grade II was obtained from Boehringer Mannheim Canada, Dorval, Quebec. Alcohol oxidase (yeast) (AO) [EC 1.1.3.13] was purchased from Provesta Corporation, Bartlesville, OK. Alcohol dehydrogenase (ADH) [donor: NAD oxidoreductase; EC 1.1.1.1] was purchased from Boehringer Mannheim Canada Ltd. The enzyme activities quoted are those of the supplier. Unit definitions are as follows: POD, one unit is the amount that will catalyze the oxidation of 1 µmole of guaiacol by hydrogen peroxide per min at 25°C at pH 7.0; AO, one unit of activity catalyzes the formation of one micromole aldehyde and hydrogen peroxide per minute in an air-saturated solution at pH 7.5, 25°C; ADH, one unit

FIGURE 8

SCHEME FOR THE ASSAY OF ETHANOL

Legend

Shown in Figure 8 is the reaction of ethanol with alcohol oxidase in the presence of oxygen to form peroxide, which can be coupled to HRP, 4-AAP, HDCBS to form a quinon-imine dye (red) detectable at 510 nm.

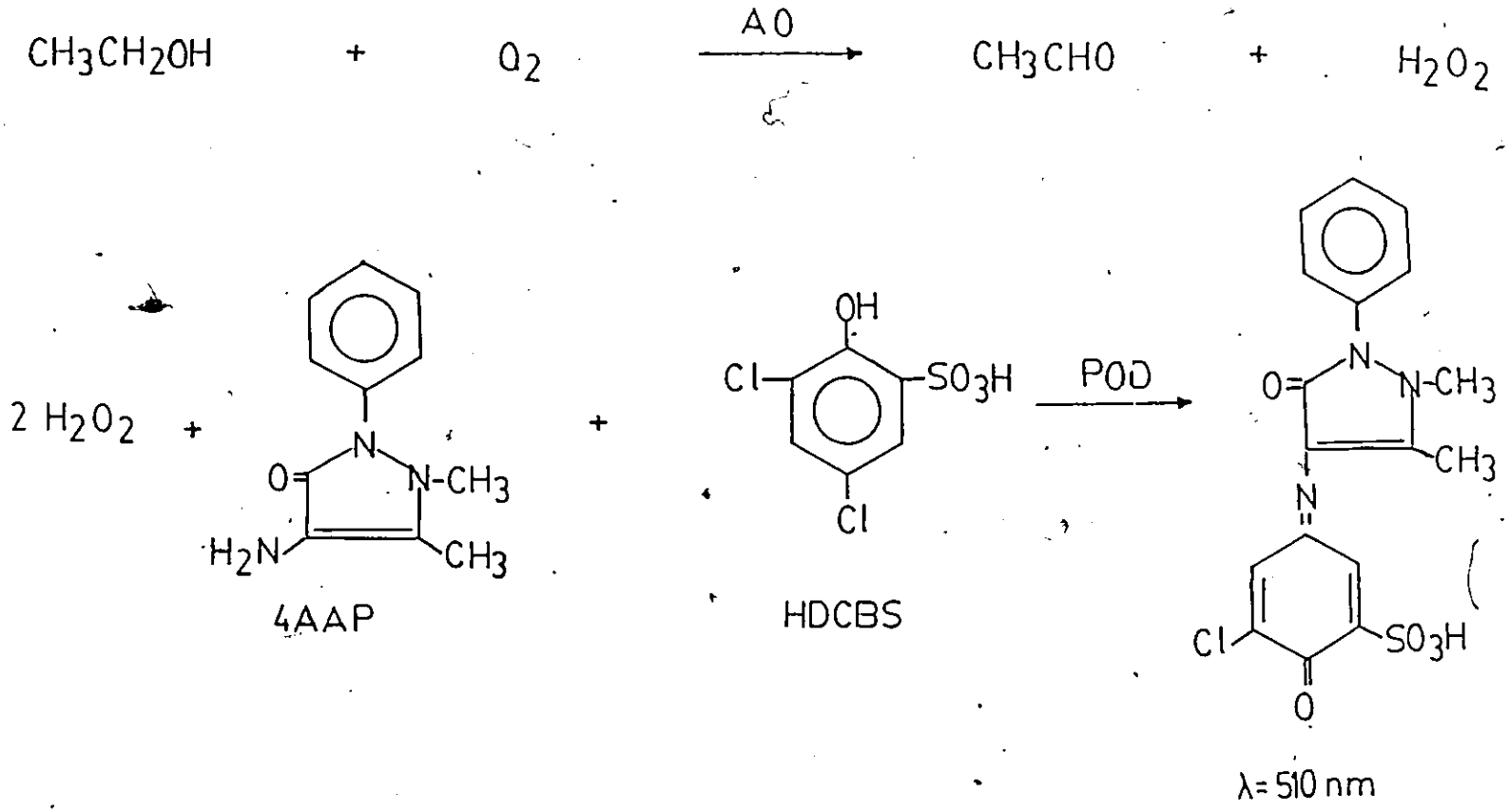

CH3CH2OH + O2 AO CH3CHO + H2O2
2 H2O2 + + OH Cl SO3H HDCBS POD
O N N-CH3 H2N CH3 4AAP
O N N-CH3 CH3 N= Cl SO3H O
λ= 510 nm

FIGURE 8

will convert 1.0 μmole of ethanol to acetaldehyde per minute at pH 8.8, 25°C.

Nylon tubing (1 or 1.5 mm bore) was obtained from Portex Limited, Hythe, Kent, England.

Triethyloxonium tetrafluoroborate was purchased from Fluka Chemical Corp., Hauppauge NY. Dichloromethane required for activation of the support was obtained from Fisher Scientific (ACS grade), Fair Lawn NJ, and supplied to us after drying over calcium hydride by Dr. D. Stephan and his research group.

The analytical grade reagents purchased from Aldrich Chemical Co., Milwaukee WI, were: 4-aminoantipyrene (AAP); and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS).

Calcium chloride, hydrogen peroxide (30% w/v), and methanol were obtained from Fisher Scientific Co. Ethylacetimidate hydrochloride and alcohol dehydrogenase were purchased from Sigma Chemical Co. St. Louis, Miss.

C. REAGENTS

All aqueous solutions were prepared using deionized distilled water. The following buffers and solutions were used:

Glycine buffer:	0.1 M, pH 8.5-11.5
Phosphate buffer:	0.1 M, pH 7.5
Borate buffer:	0.1 M, pH 8.0-10.5
Bicarbonate buffer:	0.1 M, pH 8.5-11.0

Stock Solutions:

Stock hydrogen peroxide: A 10 mM stock solution was prepared in distilled water on the day of the assay.

Ethanol standard solution: A 100 mM stock solution was prepared from absolute ethanol which had been dried over calcium hydride. Dilutions were made in distilled water.

Imidate standard solution: Stock solutions were prepared from ethyl acetimidate hydrochloride in 0.1 M glycine buffer, pH 9.5. The pH of the solution was readjusted to pH 9.5 and the imidate incubated in buffer solution for at least half-an-hour before use.

Enzyme Solutions:

Peroxidase: This solution was prepared to contain 60 U/mL POD in 0.1 M phosphate buffer, pH 7.5.

Alcohol Oxidase: 200 U/mL AO was prepared in 0.1 M phosphate buffer, pH 7.5.

Chromogen Stock Solutions:

HDCBS: A solution with a concentration of 18 mM HDCBS in 0.1 M phosphate buffer, pH 7.5 was prepared. This solution is stable for one week when stored in the dark at 4°C.

4-AAP: This solution was prepared to contain 4.8 mM 4-aminoantipyrene in 0.1 M phosphate buffer at pH 7.5. This solution is stable for several week when stored in the dark at 4°C.

Alkylating Reagent:

A stock solution of triethyloxonium tetrafluoroborate in dry dichloromethane was prepared immediately before use. This compound is extremely hygroscopic, and care must be exercised to ensure that all glassware and solvents are dry to avoid dogradation of the material to ethanol and diethyl ether. To this end, all reaction vessels were dried at 100°C and cooled to room temperature in a desiccator. In addition, dichloromethane was redistilled over calcium hydride and the nylon tubing dried for at least a day over phosphorus pentoxide under vacuum. No attempts were made to prepare exact weights of TEOTFB. An aliquot approximating the desired weight was taken, the weight recorded and dilutions prepared in dry solvent as quickly as possible. The concentration was checked with alcohol determination.

D. INSTRUMENTATION

All spectrophotometric measurements and recordings were made on the 8451A Diode Array Spectrophotometer with 7470 Plotter from Hewlett Packard, Palo Alto, CA, the Shimadzu Recording Spectrophotometer UV-240 from Shimadzu Corporation, Kyoto, Japan or the Beckman 35 UV-visible Spectrophotometer available from Beckman Instruments Inc., Irvine, CA.

All pipetting was done using Pipetman pipetters from Mandel Scientific Company Ltd., Rockwood, Ontario.

Verification of pH measurements were completed on a Fisher

Accumet pH meter, Model 800.

Weights were measured on a Mettler AE160 electronic balance.

E. METHODS

1. O-Alkylation of Nylon Tubing

Nylon-6 tubing, 1 or 1.5 mm bore was perfused with a mixture containing 18.6% w/w calcium chloride and 18.6% w/w water in methanol and heated at 60°C for 20 minutes. This process etches the inside of the nylon tubing, removing amorphous nylon and increasing the surface area available for coupling. Because of the solvent used, it was critical to remove all methanol even though it is a poorer substrate for alcohol oxidase than ethanol. To this end, the etched tubing was perfused with at least two litres of distilled water to remove methanol, and the eluate assayed until no more alcohol was present. The tubing is then dried for two days under vacuum in a desiccator over phosphorous pentoxide.

The dried tubing was filled with triethyloxonium tetrafluoroborate solution in dichloromethane and incubated at room temperature for 50 minutes.

2. Derivatization

At the end of the incubation period, unreacted alkylating reagent was removed and assayed for unreacted TEOTFB.

The tubing was then washed with dry dichloromethane. Segments of the tubing (15-cm long) were cut, and the tubing filled with 0.1 M glycine buffer, pH 10.5, for 30 minutes. The tubes were then drained and the eluate collected and assayed for ethanol. Ethanol produced from hydrolysis of the O-alkylated nylon, was calculated as bound TEOTFB. Unbound and initial concentrations of TEOTFB were assayed by hydrolysis in an aqueous solution to produce the alcohol.

3. Ethanol Assay

Ethanol produced by hydrolysis of the imidate salt was measured using alcohol oxidase and horseradish peroxidase with final assay concentrations of 10 U/mL and 3 U/mL, respectively, in 0.1 M phosphate buffer, pH 7.5. This is coupled to a chromogen system using 4-AAP and HDCBS at final assay concentrations of 2.4 mM and 9 mM, respectively, and the absorbance was detected at 510 nm.

F. RESULTS AND DISCUSSION

1. Development of an Ethanol Assay

Initial studies to measure ethanol production were focused on a commercial kit available from Sigma Chemical Company employing alcohol dehydrogenase and nicotinamide adenine dinucleotide (NAD^+). It was doubtful from the onset whether this reaction would be suitable for the purposes of an analytical tool to determine functional group density.

The main concern was that the sensitivity of this assay might be insufficient for our purposes since the chromogen has an extinction coefficient of 6220 $M^{-1}cm^{-1}$ at 340 nm (93). In addition, the equilibrium favours the reverse reaction, and as such a trapping agent, i.e., semicarbazide, must be employed to remove the aldehyde and pull the reaction in the forward direction. Preliminary attempts to optimize this assay by increasing the semicarbazide concentration showed that a 50 mM solution resulted in only 73% yield (Table III). Concentrations of trapping agent this high were difficult to dissolve. In addition, the hydrochloride lowers the pH of the solution which would require high ionic strength buffers. Since this assay results in less than quantitative yields at the ethanol step, coupling of this reaction to a hydrolysis step was expected to result in even lower yield and therefore, work with this system was discontinued.

Alcohol oxidase lacks many of the problems associated with the dehydrogenase assay: (i) the equilibrium is in the forward direction, therefore, the reaction requires no trapping agent; (ii) the product of the reaction, hydrogen peroxide, is a substrate that can be coupled to a more sensitive system using peroxidase and the chromogens 4-AAP and HDCBS. Using this coupled enzyme system, a novel and sensitive assay for the determination of ethanol was developed.

TABLE III

EFFECT OF SEMICARBAZIDE CONCENTRATION[a] ON RECOVERY OF ETHANOL

Semicarbazide mM	% Yield[b]
-	21
0.33	34
3.3	62
10.0	73
50.0	77

[a]Runs were formulated in an assay volume of 2 mL and the reaction mixture contained 0.6 mM NAD, 50 U/mL ADH and 12.5 µM ethanol, and performed in duplicates in 0.1 M glycine buffer, pH 9.0.
[b]Yields are calculated based on absorbance at 340 nm and a molar extinction coefficient of 6220.

The first step in this method development, was the determination of the optimum alcohol oxidase concentration for an end-point determination within fifteen to twenty minutes. Figure 9 shows that an alcohol oxidase concentration of 10U/mL gave an end point within the desired time, with higher concentrations being only slightly faster.

Ethanol standard curves in the range of 2 to 20 µM (Figure 10), developed using this system, showed that the assay had a molar extinction coefficient of about 20,000 at 510 nm, which is a three- to four-fold increase in sensitivity over the ADH/NAD^+ assay. This method was used in all subsequent work to determine the concentration of TEOTFB solutions and to quantitate imidates in solution or on a solid phase.

2. Imidate Hydrolysis

The reaction of triethyloxonium tetrafluoroborate with the nylon results in the formation of an ethyl imidate salt. The hydrolysis of the imidate has been shown (90,91) to be favoured at high pH's. With this in mind, a suitable pH and buffer for quantitative hydrolysis to the alcohol was sought. A 25 µM solution of ethyl acetimidate, was incubated in 100 mM borate, bicarbonate and glycine buffers in the pH range from 8-11.5, for at least an hour to allow for total conversion of the imidate to the alcohol. Other buffers, such as Tris and phosphate were also considered, but these

FIGURE 9

OPTIMUM CONCENTRATION OF ALCOHOL OXIDASE

Legend

Shown in Figure 9 is the absorbance at 510 nm versus time (minutes) for alcohol oxidase concentrations in the range 1-20 U/mL. The reaction was performed with duplicates in 0.1 M phosphate buffer, pH 7.5, with 2.4 mM 4-AAP, 9 mM HDCBS, 3 U/mL HRP and 20 µM ethanol.

Concentrations of alcohol oxidase are as follows:

—·— 1 U/mL, - - - - - 4 U/mL, ——— 10 U/mL,

—·— 16 U/mL ——— 20 U/mL

FIGURE 9

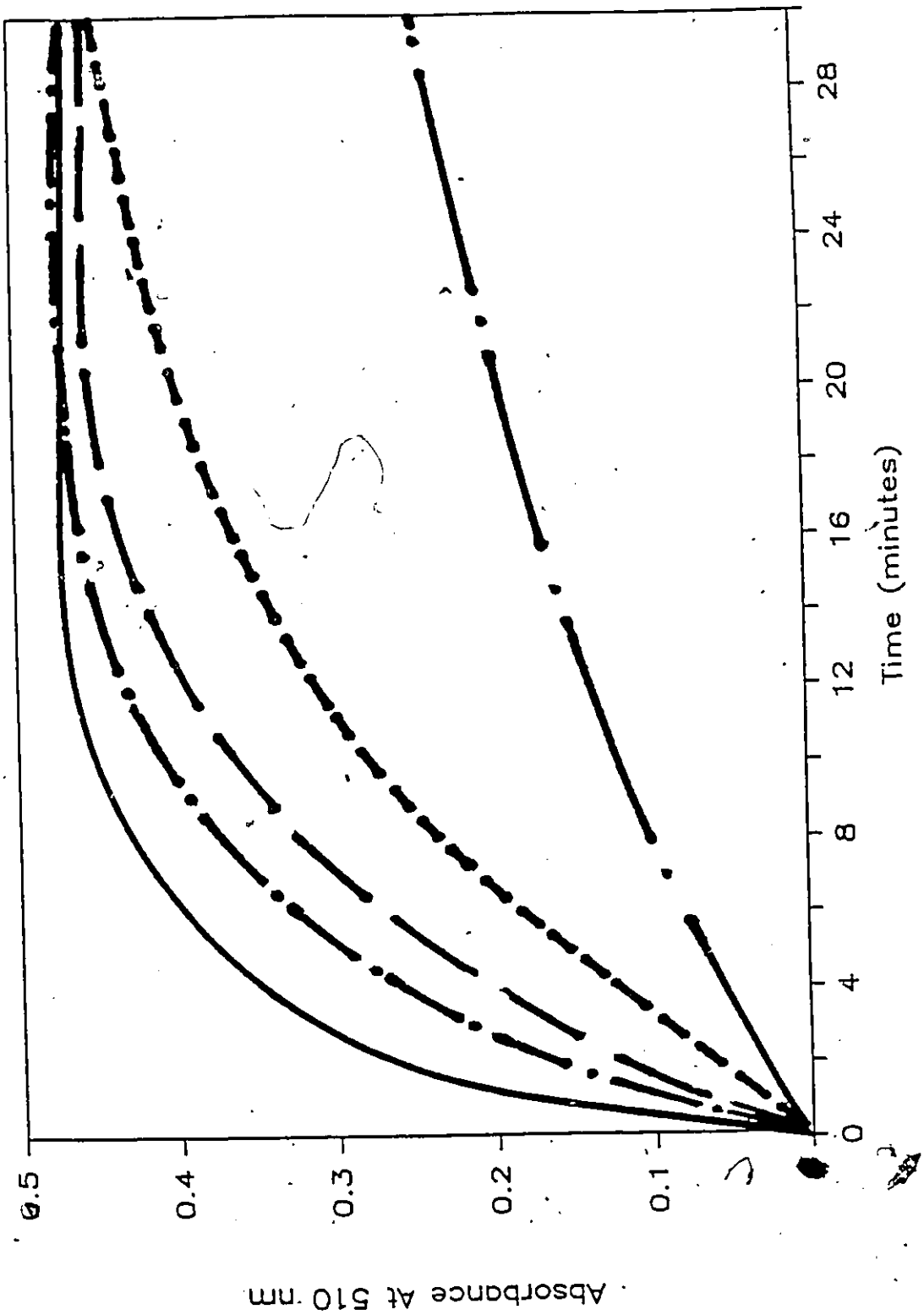

Absorbance At 510 nm
0.5
0.4
0.3
0.2
0.1
0
4
8
12
16
20
24
28
Time (minutes)

FIGURE 10

ETHANOL STANDARD CURVE

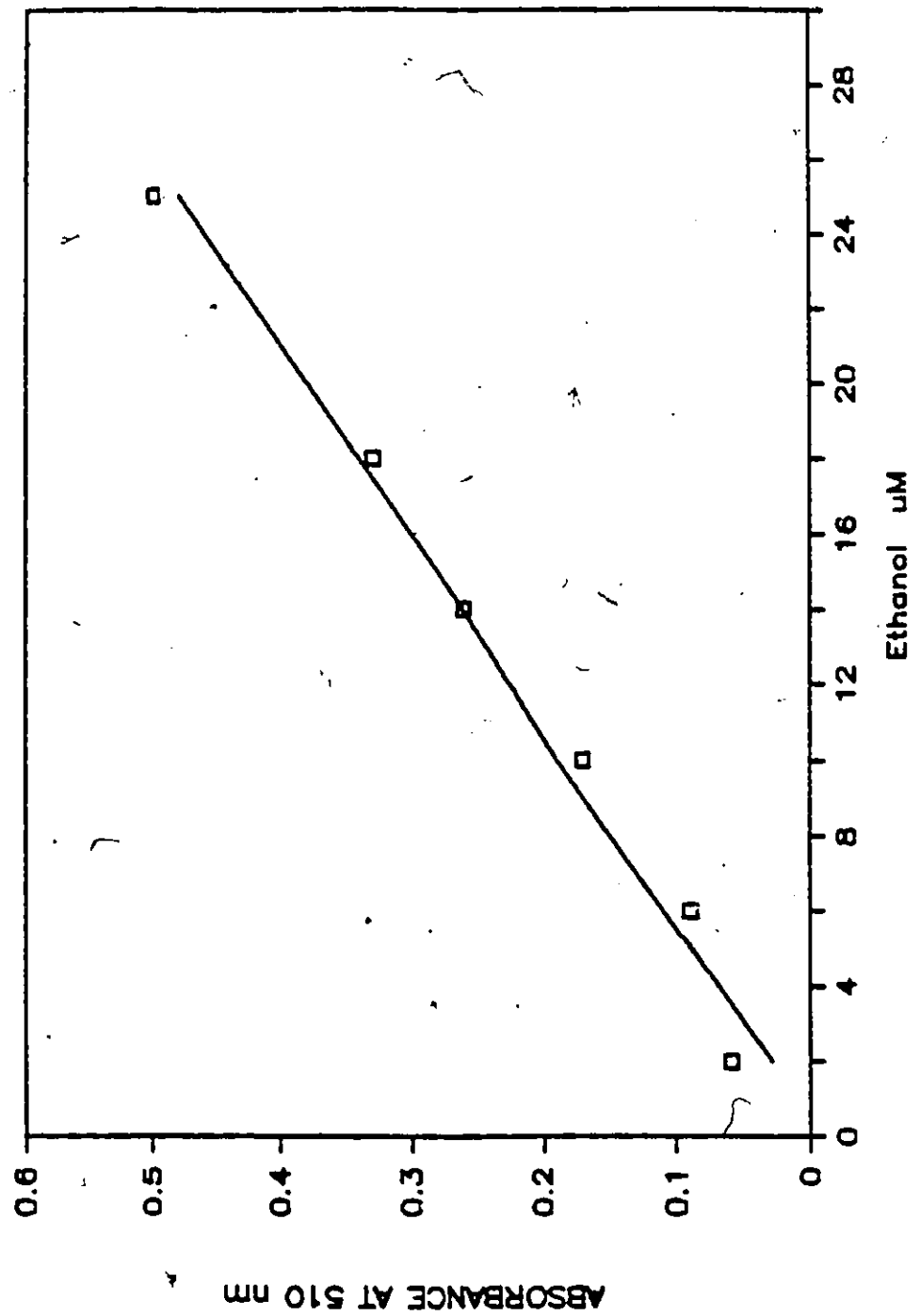
Legend

Shown in Figure 10 is the absorbance at 510 nm versus ethanol concentration between 2 and 25 μM. The alcohol oxidase-peroxidase reaction was carried out in duplicates, in 0.1 M phosphate buffer, pH 7.5 in a 1-cm pathlength cell, as described in the METHODS, p.68.

Readings were taken after 15 minute incubation at room temperature.

Linear regression analysis of this data indicated a slope of $1.955 \times 10^{-2} \pm 1.248 \times 10^{-4}$, a y-intercept of -0.0094 ± 0.0044 and a correlation coefficient of 0.9920.

FIGURE 10


ABSORBANCE AT 510 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
8
12
16
20
24
28
Ethanol uM

buffers lack the buffering capacity of the above buffers in the same pH range.

The percentage of recoveries for imidate hydrolysis (Figure 11), shows that hydrolysis is greater in the more basic pH's, in agreement with Pletcher et al., (90). The optimum pH for hydrolysis was 10.0 and 10.5 for borate and bicarbonate buffers, respectively. Hydrolysis in glycine buffer, however, appeared to be pH independent and showed a more even trend with a slight optimum at pH 9.0. This might be explained by the formation of ethanol through amidine formation (Figure 7). In addition, the percent maximum recoveries of the hydrolysis of ethyl acetimidate is nearly quantitative in glycine compared to borate and bicarbonate buffers. For these reasons subsequent hydrolysis was carried out in 0.1 M glycine buffer, pH 9.0.

An imidate standard curve in the range of 2 to 16 mM in 0.1 M glycine buffer, pH 9.5, (Figure 12), shows a linear response with quantitative recovery of ethanol.

3. Derivatization of Nylon

This work was aimed at performing the assay for functional group density on a section of tubing representative of the entire length. The immobilization of enzymes on nylon tubing, is often performed on 1-m lengths of tubing. Since the hydrolysis of the imidate salt of the nylon results in a product which is unsuitable for further derivatization and

FIGURE 11

PERCENTAGE OF RECOVERIES OF IMIDATE

IN VARIOUS BUFFERS

Legend

Shown in Figure 11 is the percent recovery of ethyl imidate in 0.1 M glycine, borate and bicarbonate buffers over the pH range of 8 to 11.5.

The reaction which was performed in duplicates, was initiated by incubation of a 20 uM sample of imidate incubated in the buffer solutions for thirty minutes. The percent recoveries are calculated on the basis of the theoretical yield of imidate hydrolysis to ethanol as measured in the standard assay using absorbance at 510 nm and a molar extinction coefficient of 21,000.

FIGURE 11

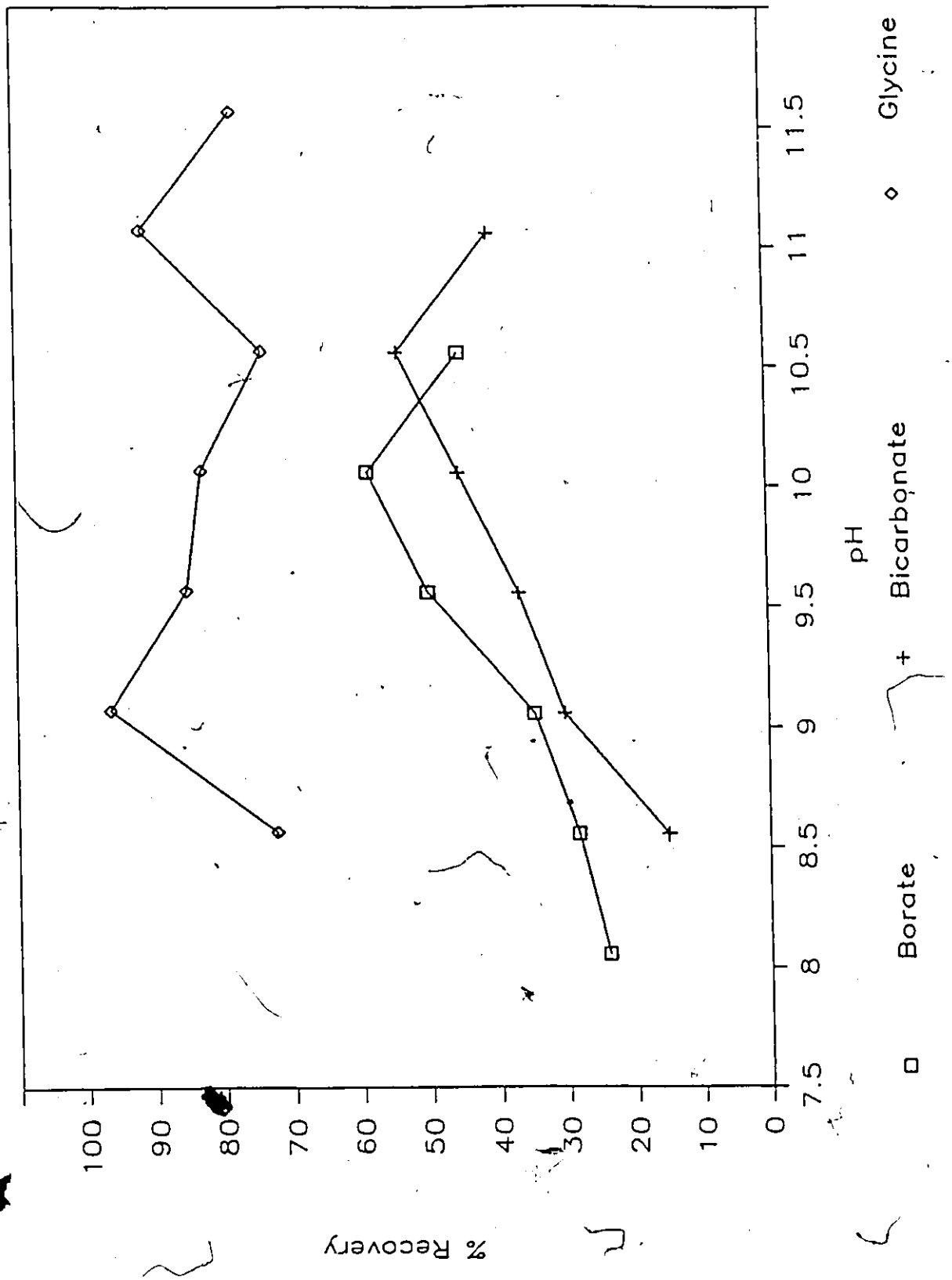

% Recovery
100
90
80
70
60
50
40
30
20
10
0
7.5
8
8.5
9
9.5
10
10.5
11
11.5
pH
Glycine
Bicarbonate
Borate

FIGURE 12

IMIDATE STANDARD CURVE

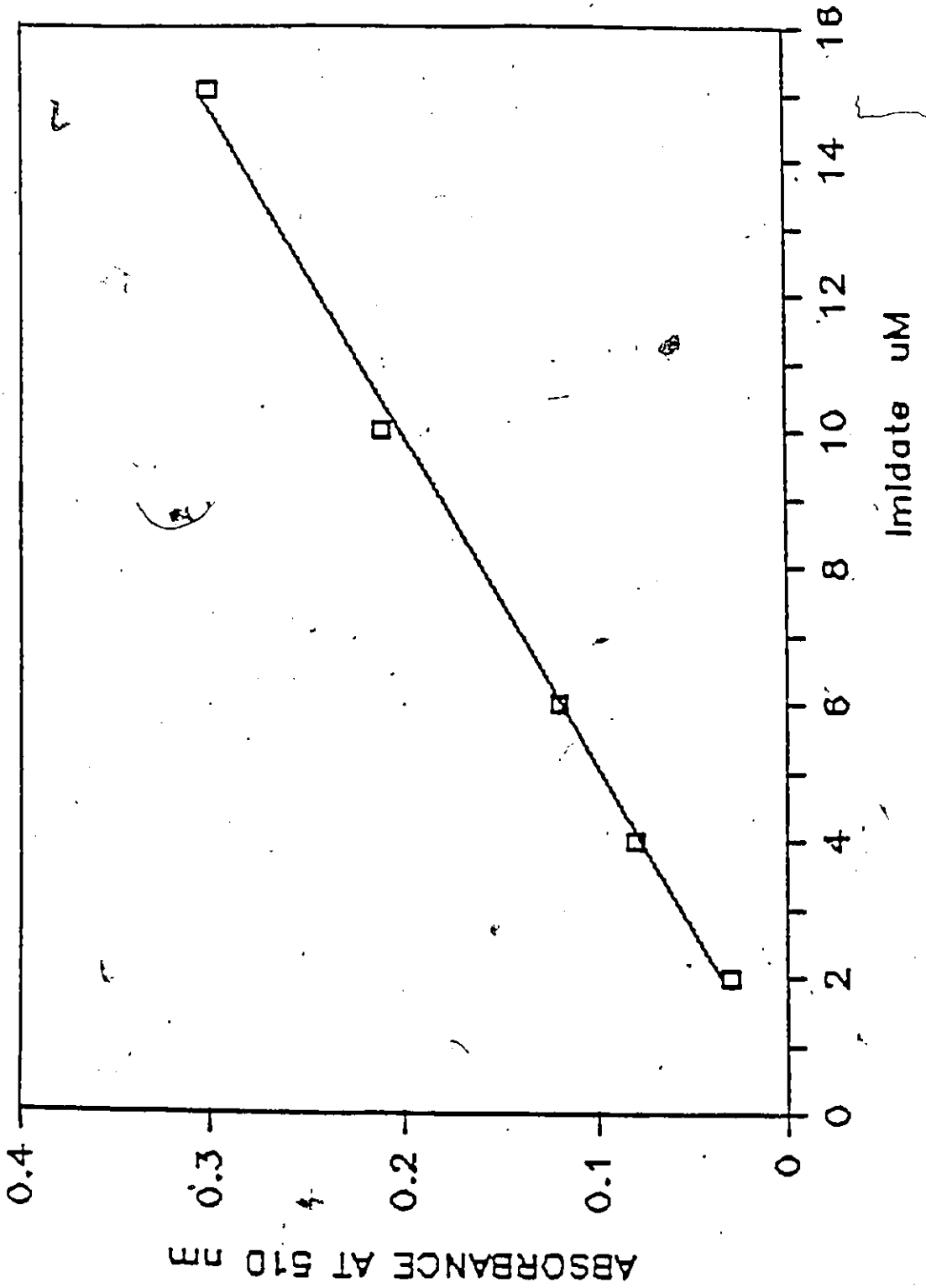
Legend

Shown in Figure 12 is the absorbance at 510 nm versus imidate concentration between 2 and 16 μM. A stock solution of imidate was incubated in 0.1 M glycine buffer, pH 9.5 for thirty minutes, afterwhich an aliquot was removed and added to the alcohol assay mixture. The reaction which was performed in duplicates, was carried out in 0.1 M phosphate buffer, pH 7.5 in a 1 cm pathlength cell as described in the METHODS, p. 67.

Readings were taken after 15 minute incubation at room temperature.

Linear regression analysis of this data indicated a slope of $2.063 \times 10^{-2} \pm 3.63 \times 10^{-4}$, a y-intercept of -0.0049 ± 0.0001 and a correlation coefficient of 0.9995.

FIGURE 12



as such involves the sacrificing of the tubing, it was desirable to use no more than 20% of the 1-m length. This corresponded to the 10 to 15-cm lengths of tubing used in this work; shorter lengths were found to be awkward to deal with.

Previous work from this laboratory (26), has shown that using a 200-uM solution of TEOTFB, i.e., limiting concentrations, the amount of coupled enzyme was comparable to that achieved with saturating concentrations (24). For this reason, the study was focused on the development of a method to assay for functional group density in the micromolar range.

The assay for imidate formation on nylon tubing was carried out by first measuring the initial concentration of the alkylating agent. Due to the hygroscopic nature of the solid, the concentration of the alkylating agent calculated by weight is not necessarily true. In addition, serial dilutions in different glassware does possess a limitation in that hydrolysis of the chemical may be progressively compounded. Attempts in limiting this factor were made by performing all experiments under dry conditions.

Unreacted alkylating agent as well as the initial solution of TEOTFB, were assayed by hydrolyzing an aliquot to produce ethanol in an aqueous solution for 20 minutes, then removing an aliquot of this solution and adding it to the assay mixture. The concentration of TEOTFB, whether

initial or unbound, represents the maximum concentration of alkylating agent.

The effect of incubation time of TEOTFB for enzyme immobilization has been previously looked at by Boss (26). Her results show that increasing the incubation time beyond 50 minutes resulted in no further increase, and in fact a decrease, in the amount of coupled enzyme, showing that no additional alkylation occurred. Morris et al., (24) have observed similar results after a 10-minute incubation. The latter authors were, however, employing saturating concentrations of TEOTFB corresponding to a 1 M solution (24). This work also looked at the effect of incubation time, but at the activation step. Table IV shows that using the same stock solution, incubation for a period of 50 or 100 minutes, showed no increase in bound alkylating agent; in fact, a decrease was seen, in agreement with previous work (26). The recovery of bound agent at the 50-minute incubation period was quantitative; however, that of the 100-minute incubation period showed a loss of 13%.

Table V shows the effect of "initial" concentration of TEOTFB on alkylation. The sum of the bound plus unbound alkylating agent is approximately equal to the initial concentration of the alkylating agent. The recovery of ethanol from the imidate hydrolysis, therefore, is near quantitative, with recoveries greater than 90%. Note, however, the huge discrepancy betweeen nominal and actual

TABLE IV

EFFECT OF INCUBATION TIME ON IMIDATE HYDROLYSIS

	Ethanol liberated	
	50 min	100 min
Initial TEOTFB		
µM[a]	10.04	10.04
(nmmol/m)[b]	(10.04)	(10.04)
Unbound TEOTFB		
µM[a]	5.47	4.76
(nmol/m)[b]	(5.47)	(4.76)
Hydrolysate		
µM[a]	4.76	3.96
(nmol/m)[b]	(4.76)	(3.96)
% Recovery	101.60	86.90

[a]Concentrations were calculated using absorbance at 510 nm and an extinction coefficient of 21,000 $M^{-1}cm^{-1}$, and the reaction was performed in triplicates on 15-cm lengths of nylon tubing, as described in the METHODS, p. 67. Concentrations calculated are those of the fluid which filled the tube, (approximately 150 µL).

[b]Numbers in brackets are expressed as nmol of ethanol liberated per metre length/1.5 mm diameter nylon tubing.

TABLE V

Effect of the "Initial" TEOTFB Concentration on Alkylation

	Ethanol Liberated	
	"100 uM[b]"	"200 uM[b]"
Initial TEOTFB		
µM[b]	21.76	26.40
(nmmol/m)[c]	(21.76)	(26.40)
Unbound TEOTFB		
µM[b]	9.52	12.00
(nmol/m)[c]	(9.52)	(12.00)
Hydrolysate		
µM[b]	10.21	12.22
(nmol/m)[c]	(10.21)	(12.22)
% Recovery	90.10	91.70

[a]Separate stock solutions of TEOTFB were prepared.
[b]Concentrations were calculated using absorbance at 510 nm and an extinction coefficient of 21,000 $M^{-1}cm^{-1}$, and the reaction was performed in triplicates on 15-cm lengths of nylon tubing, as described in the METHODS, p. 67. Concentrations calculated are those of the fluid which filled the tube, (approximately 150 µL).
[c]Numbers in brackets are expressed as nmol of ethanol liberated per metre length/1.5 mm diameter nylon tubing.

concentrations which indicates the need for accurate analytical methods for characterization of reagent solutions. In addition, comparison of hydrolysates from Tables IV and V shows that approximately 2-fold higher concentration in reagents results in aprroximately 2-fold higher level of activation, that is, a dose-response curve.

CHAPTER V

SUMMARY AND CONCLUSIONS

A general overview of the various means of immobilization and the application of this technology in the clinical laboratory has been presented. The success of immobilized enzymes in routine clinical use lies in their ability to function in automated systems. This has been shown by the incorporation of immobilized enzyme reactors into continuous flow systems e.g. Technicon AutoAnalyzer™. Immobilized enzyme technology also has the potential of being incorporated into instruments such as the BMC Hitachi™ or Coulter Dacos™, having discrete analyzers and also in systems with reusable cuvettes. As shown in Table II, a number of supported enzymes which have been incorporated into continuous-flow analyzers, have enzymes immobilized onto nylon supports. Generally, after a pitting procedure to remove amorphous nylon, the support is alkylated with either dimethyl sulphate or TEOTEB, after which arms may be attached and the enzyme coupled.

The majority of work on enzyme immobilization in clinical chemistry has focused on improvements in standard immobilization methods and comparison of these methods with soluble enzyme systems. In particular, most of the work has dealt with the immobilization of glucose oxidase/peroxidase or hexokinase/G6PDH and urease for the assay of glucose and

urea, respectively. Other work has dealt with the studies of the kinetics and stability of these enzyme preparations. Little attention however, has focused on methods to quantitate the extent of activation of the support, which might be important in the expression of enzyme activity. The aim of the present study, focusing on nylon, was to develop a method which could provide an indication of the available functional group density after the activation step. In the immobilization procedures, the amount of coupled enzyme is generally determined at the end of the procedure. If the amount of coupled enzyme is too low, the marketability of the preparation is low, representing a loss of invested time and money. If the degree of support derivatization could be measured from the onset, a poorly activated or "bad batch" of nylon could be removed from subsequent derivatization i.e., immobilization of the costly enzyme. This method is not only applicable to nylon, but also to other support matrices, particularly those which can be activated through an imidate salt. A natural extension of this work would be to couple enzyme(s) to the support and correlate the support activation with final enzyme activity. In addition, methods could be developed to assay for distal functional groups after the attachment of spacer arms at the activation sites.

APPENDIX I

CALCULATION OF FUNCTIONAL GROUP DENSITY ON NYLON SURFACES:

1. Surface area available on support = $2\pi rL$

if, Length, L = 1.0 m

Radius, r = 0.75 mm

= 4.71×10^{-3} m^2

then, Surface area = 4.71×10^{17} $Å^2$

Assuming that $CaCl_2$ etching increases the surface area ten fold then, the support surface area becomes 4.71×10^{18} $Å^2$.

2. For spacing of activation sites at 1 per 2000 $Å^2$ calculated in 1, above would be covered by:

$$\frac{4.71 \times 10^{18}}{2 \times 10^3} = 2.35 \times 10^{15} \text{ molecules}$$

$$= \frac{2.35 \times 10^{15}}{6.02 \times 10^{24}}$$

$$\sim 4 \text{ nmol}$$

This would amount to a surface density of 83 nmol/m^2.

REFERENCES

1. Trevan MD. Immobilized enzymes. An introduction and applications in biotechnology. Pp. 1-9 Toronto:John Wiley and Sons, 1980.

2. Klibanov AM. Immobilized enzymes and cells as practical catalysts. Science 1983;219:722-7.

3. Goldstein L. Immobilized enzymes. Synthesis of a new type of polyanionic and polycationic resins and their utilization for the preparation of water insoluble enzyme derivatives. Biochim Biophys Acta 1973;315:1-17.

4. Gary DN, Keyes MH, Watson B. Immobilized enzymes in analytical chemistry. Anal Chem 1977;49:1067-77.

5. Pedersen H, Horvath C. Open tubular heterogeneous enzyme reactors in continuous-flow analysis. Appl Biochem Bioeng 1931;3:2-92.

6. Werner M. Immobilized enzymes. Clin Biochem 1979;12: 200-1.

7. Lasch J, Bessmertnaya L, Kozlov LV, Antonov VK. Thermal stability of immobilized enzymes, circular dichroism, fluorescence and kinetic measurements of α-chymotrypsin attached to soluble carriers. Eur J Biochem 1976;63:591-8.

8. Bickerstaff GF. Enzymes in industry and medicine. In: New studies in biology. Pp. 7-19 Baltimore: Edward Arnold, 1986.

9. Carr PW, Bowers LD. Immobilized enzymes in analytical and clinical chemistry. In: Elving PJ, Windefordner JD. Eds. Vol. 56. Pp. 149-98 Toronto:John Wiley and Sons 1980.

10. Engasser JM, Horvath C. Diffusion and kinetics with immobilized enzymes. Appl Biochem Bioeng 1976;1:127-220.

11. Axen R. Methods for preparing immobilized enzymes. In: Salmona M, Saronio C, Garattini S. Eds. Insolubilized enzymes. Pp. 9-14 New York:Raven Press 1974.

12. Messing RA. Adsorption and inorganic bridge formation. Meth Enzymol 1976;44:148-69.

13. Solomon, B, Levin Y. Studies on the binding of amyloglucosidase to inert proteins. Biotechnol Bioenerg 1974;26:1393-8.

14. Sundaram PV. The kinetic properties of microencapsulated urease. Biochim Biophys Acta 1973; 321: 319-28.

15. Johannson A, Mosbach K. Acrylic copolymers as matrices for the immobilization of enzymes. I. Covalent binding or entrapping of various enzymes to bead-formed acrylic copolymers. Biochim Biophys Acta 1974;370:339-47.

16. Guilbault GG, Das J. Immobilization of cholinesterase and urease. Anal Biochem 1970;33:341-55.

17. Scouter WH. A survey of enzyme coupling techniques. Meth Enzymol 1987;135:30-65.

18. Weetall HH. Enzymes immobilized on inorganic supports. Trends in Biotechnol 1985;3:276-80.

19. Axen R, Ernback S. Chemical fixation of enzymes to cyanogen halide activated polysaccharide carriers. Eur J Biochem 1971;18:351-60.

20. Kohn J. Wilchek M. 1-Cyano-4-dimethylamino pyridinium tetrafluoroborate as a cyanylating agent for the covalent attachment of ligand to polysaccharide resins. FEBS Lett 1983;154:209-10.

21. Sundaram PV, Hornby WE. Preparation and properties of urease chemically attached to nylon tube. FEBS Lett 1970;10:325-7.

22. Goldstein L, Freeman A, Sokolovsky M. Chemically modified nylons as supports for enzyme immobilization. Biochem J 1974;143:497-509.

23. Campbell J, Hornby WE, Morris DL. The preparation of several new nylon tube-glucose oxidase derivatives and their incorporation into the "reagentless" automated analysis of glucose. Biochim Biophys Acta 1975;384:307-16.

24. Morris DL, Campbell J, Hornby WE. A chemistry for the immobilization of enzymes on nylon. Biochem J 1975; 147:593-603.

25. Zaborsky OR. Immobilized enzymes - miscellaneous methods and general classification. Meth Enzymol

1976;44:317-32.

26. Boss S. The immobilization and clinical evaluation of enzymes on nylon tubing modified with long spacer arms: peroxidase, glucose oxidase and oxalate oxidase. Doctoral Dissertation, University of Windsor 1986.

27. Sung C, Lavin A, Klibanov AM, Langer R. An immobilized enzyme reactor for the detoxification of bilirubin. Biotechnol Bioenerg 1986;28:1531-9.

28. Bourget L, Chang TMC. Phenylalanine ammonia-lyase immobilized in semipermeable microcapsules for enzyme replacement in phenylketonuria. FEBS Lett 1985;180:1-8.

29. Kobos RK. Enzyme-based electrochemical biosensors. Trends in Anal Chem 1987;6:6-9.

30. Guilbault GG, Kauffmann JM. Enzyme-based electrodes as analytical tools. Biotechnol Appl Biochem 1987;9:95-113.

31. Updike SJ, Hicks GP. The enzyme electrode. Nature 1967;214:986-8.

32. Lubrano GJ, Guilbault GG. Glucose and L-amino acid electrodes based on enzyme membranes. Anal Chim Acta 1978;97:229-36.

33. Moody GJ, Sanghera GS, Thomas JDR. Amperometric enzyme electrode system for the flow injection analysis of glucose. Analyst 1986;111:605-9.

34. Yao T. A chemically-modified enzyme membrane electrode as an amperometric glucose sensor. Anal Chim Acta 1983;148:27-33.

35. Cass AEG, Davis G, Francis GD, Hill HAO, Aston WJ, Higgins IJ, Plotkin EV, Scott LDL, Turner PF. Ferrocene-mediated enzyme electrode for amperometric determination of glucose. Anal Chem 1984;56:667-71.

36. Kulys JJ, Vicius VSAL, Pesliakiene MV, Gureviviene VV. The determination of glucose, hypoxanthine and uric acid with use of bi-enzyme amperometric electrodes. Anal Chim Acta 1983;148:13-8.

37. Mullen WH, Churchhouse SJ, Vadgama PM. Enzyme electrode for glucose based on the quinoprotein

glucose dehydrogenase. Analyst 1985;110:925-8.

38. Guilbault GG, Montalvo Jr. JG. A urea-specific enzyme electrode. J Amer Chem Soc 1969;91:2164-5.

39. Guilbault GG, Mascini M. Urease coupled ammonia electrode for urea determination in blood serum. Anal Chem 1977;49:795-8.

40. Vincke BJ, Develeeschouwer MJ, Patriarche GJ. Contributionn au developpement d'un nouveau modele d'electrode:L'electrode bacterienne. Anal Lett 1983;16:673-84.

41. Begum KD, Mottola HA. Nylon shavings enzyme reactor for batch determination of urea. Anal Biochem 1984;142:1-6.

42. Vadgama P. Urea pH electrode: characterization and optimization for plasma measurements. Analyst 1986; 111:875-8.

43. Tor R, Freeman A. New enzyme membrane for enzyme electrodes. Anal Chem 1986;58:1042-6.

44. Rahni MAN, Guilbault GG. Immobilized enzyme electrode for the determination of oxalate in urine. Anal Chem 1986;58:523-6.

45. Buffone GJ, Johnson JM, Lewis SA, Sparks JW. Evaluation of an immobilized enzyme analyzer for measuring galactose in serum. Clin Chem 1980;26: 339-40.

46. Huang H, Kuan SS, Guilbault GG. Amperometric determination of total cholesterol in serum, with use of immobilized cholesterol ester hydrolase and cholesterol oxidase. Clin Chem 1977;23:671-6.

47. Fonong T, Rechnitz GA. Enzyme electrode for the determination of salicylate. Anal Chim Acta 1984;158:357-62.

48. Nanjo K, Guilbault GG. Enzyme electrode sensing oxygen for uric acid in serum and urine. Anal Chem 1974;46:1769-72.

49. Riesel E, Katchalski E. Preparation and properties of water-insoluble derivatives of urease. J Biol Chem 1964;239:1521-4.

50. Sundaram PV, Hornby WE. Preparation and properties

of urease chemically attached to nylon tube. FEBS Lett 1970;10:325-7.

51. Sundaram PV, Iglol MP, Wasserman R, Hinsch W, Knoke KJ. Immobilized-enzyme nylon tube reactors for routine determination of urea and citrulline in serum. Clin Chem 1978;24:234-9.

52. Chirillo R, Caenaro G, Pavan B, Pin A. The use of immobilized reactors in continuous-flow analyzers for the determination of glucose, urea, and uric acid. Clin Chem 1979;25:1744-8.

53. Werner M, Mohrbacher RJ, Riendeau CJ, Murador E, Cambiaghi S. Immobilized enzymes in continuous-flow analysis. Clin Chem 1979;25:20-3.

54. Onyezili FN and Onitiri AC. Polysaccharide derivatives as coats for nylon tube urease. Anal Biochem 1981;117:121-5.

55. Onyezili FN, Onitiri AC. The effects of substrate flow-rate on immobilized urease assays. Biochim Biophys Acta 1981;659:244-8.

56. Updike GP, Hicks SJ. The preparation and characterization of lyophilized polyacrylamide enzyme gels for chemical analysis. Anal Chem 1966;38:726-30.

57. Campbell J, Chawea AS, Chang TMS. The use of immobilized enzyme-membrane sandwich reactors in automated analysis. Anal Biochem 1977;83:330-5.

58. Leon LP, Sansur M, Snyder LR, Horvath C. Continuous-flow analysis for glucose, triglycerides, and ATP with immobilized enzymes in tubular form. Clin Chem 1977;23:1556-62.

59. Inman DJ, Hornby WE. The immobilization of enzymes on nylon structures and their use in automated analysis. Biochem J 1977;129:255-62.

60. Joseph MD, Kaspizak DJ, Crouch SR. A stopped-flow clinical analyzer in which immobilized-enzyme reaction loops are used. Clin Chem 1977;23:1033-6.

61. Thompson RO, Mondoke CS, Womack JP. A procedure for immobilizing enzymes on nylon. Anal Lett 1985;18:93-107.

62. Szajani B, Molnar A, Klamar G, Kalman M. Prepara-

tion, characterization, and potential application of an immobilized glucose oxidase. Appl Biochem Biotechnol 1987;14:37-41.

63. Kuan JA, Kuan SS, Guilbault GG. Determination of plasma glucose with use of a stirrer containing immobilized glucose dehydrogenase. Clin Chem 1977;23:1059-61.

64. Sundaram PV, Blumenberg B, Hinsch W. Routine glucose determination in serum by use of an immobilized glucose dehydrogenase nylon-tube reactor. Clin Chem 1979;25:1436-9.

65. Sundaram PV, Apps DK. Preparation and properties of nylon-tube-supported nicotinamide-adenine dinucleotide kinase. Biochem J 1977;161:441-2.

66. Bisse E, Scholer A, Vonderschmitt DJ. A new method for coupling glucose dehydrogenase to glass tubes activated with titanium tetrachloride. FEBS Lett 1982;138:316-8.

67. Garber CC, Feldbruegge D, Miller RC, Carey RN. Evaluation of the coimmobilized hexokinase/glucose-6-phosphate dehydrogenase method for glucose, as adapted to the Technicon SMAC. Clin Chem 1978;24:1186-90.

68. Jablonski E, DeLuca M. Properties and uses of immobilized light-emitting enzyme systems from Beneckea harveyi. Clin Chem 1979;25:1622-7.

69. Haggerty C, Jablonski E, Star L, DeLuca M. Continuous monitoring of reactions that produce NADH and NADPH using immobilized luciferase and oxidoreductases from Beneckea harveyi. Anal Biochem 1978;88:162-73.

70. Jablonski E, DeLuca M. Immobilization of bacterial luciferase and oxido-reductase and assays using immobilized enzymes. Meth Enzymol 1978;57:202-14.

71. Kricka LJ, Wienhausen GK, Hinkley KE, DeLuca M. Automated bioluminescent assays for NADH, glucose-6-phosphate, primary bile acids, and ATP. Anal Biochem 1983;129:392-7.

72. Schoelmerich J, Roda A, DeLuca M. Immobilized enzymes for assaying bile acids. Meth Enzymol 1986;133:215-48.

73. Nicolas JC, Boussioux AM, Boularan AM, Descomps B, DePaulet AC. Bioluminescent assay of fentomole levels of estrone and estradiol. Anal Biochem 1983;135:141-5.

74. Ford J, DeLuca M. A new assay for picomole levels of androsterone and testosterone using co-immobilized luciferase, oxidoreductase, and steroid dehydrogenase. Anal Biochem 1981;110:43-8.

75. Sundaram PV, Igloi MP, Wassermann R, Hinsch W. Immobilized-enzyme nylon-tube reactor for routine determination of uric acid in serum. Clin Chem 1978;24:1813-7.

76. Salleh AB, Ledingham WM. Uricase immobilized onto nylon tubes for use in automated analysis of serum urate. Anal Biochem 1981;116:40-7.

77. Thomasson CG, Blijenberg BG, Leijnse B. Evaluation of the Technicon bound uricase method for the determination of uric acid in urine. J Clin Chem Clin Biochem 1982;20:299-303.

78. Nakamura N, Musayama K, Kinoshita T. Immobilization of uricase on protamine bound to glass beads and its application to determination of uric acid. Anal Biochem 1986;152:386-90.

79. Bais R, Potezny N, Edwards JB, Rofe AM, Conyers RAJ. Oxalate determination by immobilized oxalate oxidase in a continuous-flow system. Anal Chem 1980;52:508-11.

80. Sundaram PV, Hinsch W. Single- and couple-enzyme nylon tube reactors for routine determination of pyruvate and lactate in serum. Clin Chem 1979;25:285-8.

81. Bovara R, Carea G, Cremonesi P, Mazzola G. Continuous-flow analysis of 3.-hydroxysteroids using immobilized 3.-hydroxysteroid dehydrogenase. Anal Biochem 1981;112:239-43.

82. Danielsson B, Mattiasson B, Mosbach K. Enzyme thermistor devices and their analytical applications. Appl Biochem Bioeng 1981;3:97-143.

83. Bowers LD, Carr PW. An immobilized-enzyme flow-enthalpimetric analyzer: Applications to glucose determination by direct phosphorylation catalyzed by hexokinase. Clin Chem 1976;22:1427-33.

84. Bowers LD, Canning Jr. LM, Sayers CN, Carr PW. Rapid-flow-enthalpimetric determination of urea in serum, with use of an immobilized urease reactor. Clin Chem 1976;22:1314-8.

85. Sundaram PV. Immobilized-enzyme pipette. Biochem J 1979;179:445-7.

86. Kristalovich B. Long-arm thiolated polyacrylamide beads and their application in coupling experiments with papain. MSc Thesis, University of Windsor, 1983.

87. Dulay B. Studies on immobilization of Horseradish peroxidase. MSc Thesis, University of Windsor, 1986.

88. Harake B. Preparation and characterization of defined conjugates of glucose oxidase and peroxidase. MSc Thesis, University of Windsor, 1988.

89. Hornby WE, Goldstein L. Immobilization of enzymes on nylon. Meth Enzymol 1976;44:118-34.

90. Pletcher TC, Koehler S, Cordes EH. Concerning the mechanism of hydrolysis of N-methylacetimidate esters. J Amer Chem Soc 1968;90:7072-6.

91. Richardson CC,. Chemical cross-linking: reagents and problems in studies of membrane structures. Ann Rev Biochem 1977;46:523-31.

92. Chaturvedi RK, Schmir GL. The hydrolysis of N-substituted acetimidate esters. J Amer Chem Soc 1968; 90:4413-20.

93. Sigma Chemical Co Package Insert: Ethanol Assay. St. Louis Missouri, 1985.

VITA AUCTORIS

ERICA SULLIVAN

Birthplace:
Kingston, Jamaica, W.I.

Education:

1985 - Pres.	M.Sc. Clinical Chemistry University of Windsor Windsor, Ontario
1982 - 1984	B.Sc. Honours Biochemistry University of Windsor Windsor, Ontario
1979 - 1982	B.Sc. Biology University of Windsor Windsor, Ontario

Awards:
University of Windsor Postgraduate Scholarship (1986 - 88)
William A. Redmond Memorial Bursary (1987)
Government of Jamaica Scholarship (1972 - 78)

Professional Memberships:
American Association for Clinical Chemistry (AACC)
Chemical Society for Canada (CSC)